METABOLISM OF CRUCIFEROUS CHEMICAL DEFENSES BY PLANT PATHOGENIC FUNGI

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in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Chemistry

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by

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ABSTRACT

Plants produce complex mixtures of secondary metabolites to defend themselves from pathogens. Among these defenses are metabolites produced *de novo*, phytoalexins, and constitutive metabolites, phytoanticipins. As a counter-attack, pathogenic fungi are able to transform such plant defenses utilizing detoxifying enzymes. This thesis investigates the metabolism of two important cruciferous phytoalexins (brassinin (**33**) and camalexin (**39**)) by the phytopathogenic fungus *Botrytis cinerea* and the metabolism of cruciferous phytoanticipins (glucosinolates and derivatives) by three economically important fungi of crucifers *Alternaria brassicicola, Rhizoctonia solani* and *Sclerotinia sclerotiorum* to investigate their role in cruciferous defense.

In the first part of this thesis, the transformations of brassinin (**33**) and camalexin (**39**) by *B. cinerea* were investigated. During these studies a number of new metabolites were isolated, their chemical structures were determined using spectroscopic techniques, and further confirmed by synthesis. Camalexin (**39**) was transformed via oxidative degradation and brassinin (**33**) was hydrolyzed to indoly-3-methanamine (**49**). The metabolic products did not show detectable antifungal activity against *B. cinerea,* which indicated that these transformations were detoxification processes. Camalexin (**39**) was found to be more antifungal than brassinin (**33**).

In the second part of this thesis, the metabolism of glucobrassicin (**86**), 1-methoxyglucobrassicin (**87**), 4-methoxyglucobrassicin (**90**), phenylglucosinolate (**65**), and benzylglucosinolate (**66**), the corresponding desulfoglucosinolates and derivatives by three fungal pathogens (*A. brassicicola, R. solani* and *S. sclerotiorum*) was investigated and their antifungal activity against the same pathogens was tested. Aryl

glucosinolates **65** and **66** were metabolized by *A. brassicicola* but not by *R. solani* or *S. sclerotiorum*, whereas indolylglucosinolates were not metabolized by any pathogen. Indolyl desulfoglucosinolates (**159** and **233**) were transformed by *R. solani* and *S. sclerotiorum* to the corresponding carboxylic acids and indolyl acetonitriles **40**, **102**, and **103** were also metabolized to the corresponding carboxylic acids by all pathogens. None of the glucosinolates or their desulfo derivatives showed antifungal activity, but some of their metabolites showed low to very high antifungal activities. Among these metabolites, diindolyl-3-methane (**113**) showed the highest antifungal activity, and benzyl isothiocyanate (**170**) showed higher inhibitory effect against *R. solani* and *S. sclerotiorum*, but did not inhibit the growth of *A. brassicicola*.

The cell-free extracts of *A. brassicicola*, *R. solani*, and *S. sclerotiorum* were tested for myrosinase activity against several glucosinolates. The cell-free extracts of mycelia of *A. brassicicola* displayed higher myrosinase activity for sinigrin (**131**), phenyl and benzyl glucosinolates **65** and **66**, but lower activities for glucobrassicin (**86**) and 1-methoxyglucobrassicin (**87**); no myrosinase activity was detected in mycelia of either *R. solani* or *S. sclerotiorum.*

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Dedication:

To my family

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LIST OF ABBREVIATIONS

A. b	*Alternaria brassicicola*
Ac	Acetyl
Ac_2O	Acetic anhydride
AcOH	Acetic acid
APO2	Accumulation Of Photosystem One 2
APO3	Accumulation Of Photosystem One 3
ATP	Adenosine triphosphate
B.	*Brassica*
t-Boc	*tert*-Butoxycarbonyl
br	Broad
BGT	Brassinin glucosyltransferase
BO	Brassinin oxidase
BSA	Bovine serum albumin
^{13}C NMR	Carbon-13 nuclear magnetic resonance
calcd.	Calculated
DAD	Diode Array Detector
DCM	Dichloromethane
DME	1,2-Dimethoxyether
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
β-D-Glc	β-D-Glucose
EI	Electron impact

EI-HRMS	Electron ionization high resolution mass spectroscopy
ESI	Electron spray ionization
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FCC	Flash column chromatography
FTIR	Fourier transformed infrared
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
GST	Glutathione-S-transferase
GT	Glucosyltransferase
^{1}H NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
HR	High resolution
Hz	Hertz
IBX	2-Iodoxybenzoic acid
IGLs	Indolylglucosinolates
J	Coupling constant
m/*z*	Mass/charge ratio
MAM1	Methylthioalkylmalate synthase 1
MAM2	Methylthioalkylmalate synthase 2
MAM3	Methylthioalkylmalate synthase 3
Me	Methyl
MeI	Methyl iodide

MeOH	Methanol
MHz	Megahertz
min	Minute(s)
MM	Minimal media
MS	Mass spectrum
NAD	Nicotinamide Adenin Dinucleotide
NADH	Nicotinamide Adenin Dinucleotide Hydride
NCS	N-Chlorosuccinimide
PDA	Potato dextrose agar
R. s	*Rhizoctonia solani*
r.t.	Room temperature
t_R	Retention time
S. D.	Standard deviation
S. E.	Standard error
S. s	*Sclerotinia sclerotiorum*
TEA	Triethyl amine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UV	Ultraviolet

CHAPTER 1 : INTRODUCTION

1.1. General objectives

Cruciferous (Brassicaceae family) crops are important sources of vegetables, as well as edible and industrial oils, condiments and forage. Many well known vegetables such as cabbage (*Brassica oleracea* var. *capitata* L.), broccoli (*B. oleracea* var. *botrytis* L.), cauliflower (*B. oleracea* var. *italica* L.), Brussels sprouts (*B. oleracea* var. *gemmifera* L.), kohlrabi (*B. oleracea* var. *gongylodes* L.), Chinese cabbage (*B. campestris* var *Pekinensis* L.), turnip (*B. campestris* var. *rapifera* L.), and rutabaga (*B. napus* var. *napobrassica* L.) are part of this family (Gomez-Campo, 1999; Pua and Douglas, 2004). Oilseed crucifers (*Brassica* spp.) are the third largest source of edible vegetable oils, while brown (*Brassica juncea* L. Czern.) and white (*Sinapis alba* L.) mustard seeds, as well as wasabi (*Wasabia japonica* M.) are well-known condiments (Pedras, 1998). Cruciferous plants are also known to have anti-carcinogenic properties (Mithen, 2001; Arikawa and Gallaher, 2008).

Fungal pathogens of crucifers cause severe yield loses in many regions of the world. Common practices to prevent the spread of fungal diseases are crop rotations, use of certified seed, removal of infected stubble, and application of fungicides. Due to increasing problems and concerns over the use of fungicides, there is a great interest in chemical defenses produced by plants. Plants produce secondary metabolites that may have antifungal activity and are part of their defense mechanisms against fungal attack (Pedras et al., 2011a). Some of these compounds are biosynthesized de novo by plants in response to pathogen attack, and are known as phytoalexins (Baily and Mansfield, 1982; Brooks and Watson, 1985; VanEtten et al., 1994; Smith, 1996). It was found that

some pathogenic fungi of crucifers are able to overcome these plant chemical defenses through metabolism and detoxification by utilizing a variety of enzymatic reactions (Pedras and Ahiahonu, 2005). The metabolic detoxification of phytoalexins can potentially deplete cruciferous plants from important inducible chemical defenses and render plants susceptible to pathogenic attack. It is thus important to understand the detoxification pathway of phytoalexins by phytopathogenic fungi and to inhibit these degradation processes (Pedras and Ahiahonu, 2005). Another group of secondary metabolites produced in plants constitutively are known as phytoanticipins (VanEtten et al., 1994). The major phytoanticipins produced by cruciferous plants are the glucosinolates and their derivatives. The direct interaction between glucosinolates and derivatives with fungal pathogens is not known. In order to understand the roles of these compounds (phytoanticipins) in crucifers it is important to study the potential metabolism of glucosinolates and derivatives by plant pathogenic fungi. The project's objectives were:

(i) Synthesis and evaluation of the antifungal activity of the phytoalexins brassinin (**33**) and camalexin (**39**) against *Botrytis cinerea*. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel)

(ii) Investigation of metabolic transformation of brassinin (**33**) and camalexin (**39**) by *B. cinerea*.

(iii) Isolation, characterization, and synthesis of metabolites produced in the metabolism of brassinin (**33**) and camalexin (**39**), determination of their antifungal activities and propose metabolic pathways.

(iv) Synthesis and evaluation of the antifungal activity of indole glucosinolates and derivatives against *Alternaria brassicicola* (Schweinitz, Wiltshire), *Rhizoctonia solani* Kühn, and *Sclerotinia sclerotiorum* (Lib.) de Bary.

(v) Investigation of the metabolism of glucosinolates and derivatives by *A. brassicicola*, *R. solani*, and *S. sclerotiorum*.

(vi) Isolation and characterization of potential metabolites produced in the biotransformation of glucosinolates and derivatives and propose metabolic pathways.

1.2. Plant-pathogen interactions

Plants are under challenge by different biotic and abiotic stresses. Plant pathogenic fungi are present in plant communities, and their impacts are diverse and often severe. The interaction between plant and pathogen involves a metabolic attack-counterattack that is generally unique to a particular plant-pathogen system. Both plants and pathogens produce secondary metabolites in this attack-counterattack mechanism (Pedras and Ahiahonu 2002; Pedras and Bisenthal 2000a; 2000b; Pedras and Ahiahonu 2005; Pedras, 2011). Phytopathogenic fungi are capable of infecting plants using secondary metabolites that damage plant tissues to facilitate attack and colonization. Such secondary metabolites are named as phytotoxins, which can be host selective or non-host selective. Host-selective toxins are toxic specifically to the host plant, whereas non-selective toxins are toxic to a wider range of species including host species (Pedras et al., 2000; Pedras and Ahiahonu, 2004; Pedras and Yu, 2008). In addition to phytotoxins, phytopathogenic fungi may produce other groups of metabolites mediating plant defense response, including elicitors. Elicitors induce plant defense responses such as cell wall reinforcement, biosynthesis of chemical defenses, and pathogenesis-related proteins (Pedras and Yu, 2008). General elicitors are able to trigger defenses both in host and non-host plants, whereas race specific elicitors induce defense responses in specific hosts. General elicitors, also called microbial or

pathogen-associated molecular patterns (MAMPs or PAMPs), include lipids, lipopolysaccharides, chitin, β-glucans, and fungal-specific glycosylated proteins etc. (Dangl and Jones, 2001; Grayer and Kokubun, 2001).

To protect themselves from fungal pathogens, plants have different defense barriers including physical and chemical barriers. After detecting a pathogen's signal(s), plants produce a complex mixture of macromolecules such as callose, chitinases, glucanases and proteases and also secondary metabolites to prevent the infection (Maor and Shirasu, 2005). Plant defense metabolites can be preformed (constitutive) and inducible (phytoalexins). Constitutive defense metabolites are always present, irrespective of whether or not the plant tissue is challenged by microorganism, whereas induced metabolites are biosynthesized in response to microbial attack or other stresses (Osbourn, 1996). The preformed antifungal compounds of low molecular weight are called preinfectional metabolites, prohibitins, or phytoanticipins (Grayer and Kokubun, 2001; VanEtten et al., 1994). A large number of constitutive plant metabolites have been reported to have antifungal activity. Well-known examples include phenols and phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, and glucosinolates (Cvikrova et al. 2006; Jones et al. 2000; Halkier and Gershenzon, 2006). Phytoalexins by definition are antimicrobial secondary metabolites produced de novo in response to biotic and abiotic stresses (e.g., microbial attack, heavy metal salts, or UV radiation) (Baily and Mansfield, 1982; Smith, 1996).

1.3. Cruciferous fungal pathogens

Fungal pathogens of crucifers are categorized into distinct groups: biotrophic, hemibiotrophic and necrotrophic fungi. Hemibiotrophic and necrotrophic fungi are

destructive parasites and derive their nutrients from the dead cells of plants (Veronese et al., 2006). Their effects vary from local discrete lesions, as seen in some leaf spot diseases, to massive tissue destruction, as seen in fruit rots. Examples of major fungal pathogens of crucifers are *B. cinerea, Leptosphaeria maculans* (*Phoma lingam*), *A. brassicicola, Alternaria brassicae* (Berk.) Sacc., *R. solani,* and *S. sclerotiorum*. The biotrophic fungal parasites, in contrast to the necrotrophic parasites, can only grow on living hosts (Schulze-Lefert and Panstruga, 2003). They are termed obligate parasites that include the downy mildews (e.g., *Peronospora,* Straminipila), the powdery mildews (e.g., *Erysiphe,* Ascomycotina) and the rust fungi (e.g., *Puccinia* , Basidiomycotina) (Casimiro et al., 2006).

1.3.1. Botrytis gray mold

B. cinerea is the causal organism of gray mold of brassica crops in the field and brown soft rot of cabbage and other brassicas in storage (Geeson and Browne, 1979). Other than brassica crops, it also causes gray mold disease and great yield losses in a very large number of crops, including grapevines (*Vitis vinifera* L.), tomatoes (*Solanum lycopersicum* L.), etc. The fungus is a weak pathogen under field conditions and infects plants more readily when the tissue is damaged, for instance, by frost, insects, slugs, and farm machinery. *B. cinerea* tolerates a wide range of temperatures. Mycelium growth occurs between 0 and 25 °C, which explains why the fungus can cause damage on vegetables in cold storage. In storage, most strains of *B. cinerea* can grow at 0-2 °C, although at a slower rate (Elad et al., 2007). *B. cinerea* produces a variety of phytotoxic metabolites including oxalic acid, polyketide lactones, sesquiterpenoids, and cell-wall-degrading enzymes together with other pathogenesis related proteins. Two groups of

non-selective phytotoxins have been reported from *B. cinerea*: sesquiterpene botrydial (**1**) and related compounds (**2-6**), and botcinic acid (**7**) and botcinic acid derivatives (**8-9**) (Figure 1.1). Botrydial (**1**) is produced during plant infection and induces chlorosis and necrosis; botcinic acid (**7**) and derivatives (**8-9**) not only induce chlorosis and necrosis but also have shown antifungal activities (Dalmais et. al., 2011). Pathogenic isolates of *B. cinerea* produce an ABC transporter that increases tolerance of the pathogen to the phytoalexin camalexin (**39**) in *Arabidopsis thaliana* (L.) Heynh. (Choquer et. al., 2007).

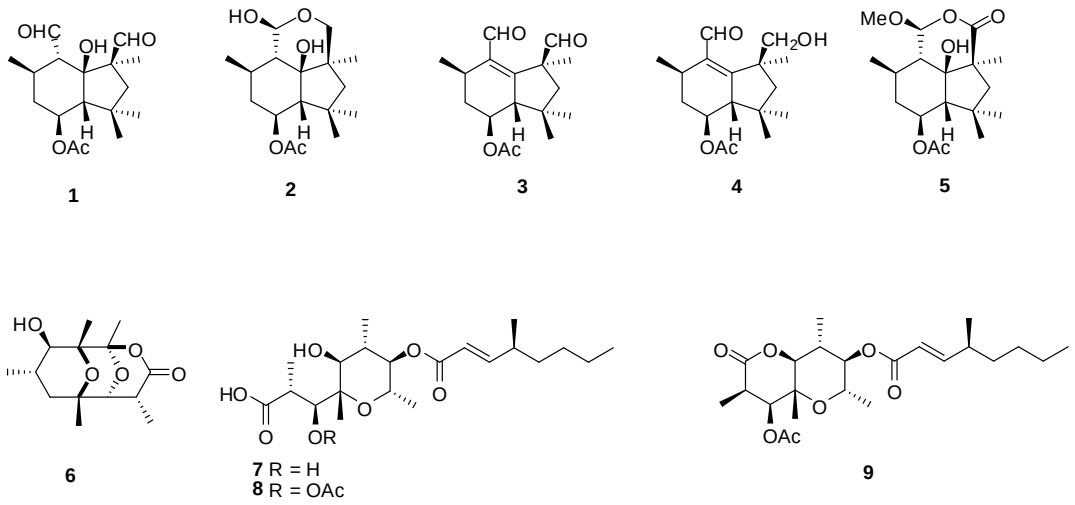


Figure 1.1 Structures of phytotoxins and derivatives from *Botrytis cinerea*: botrydial (**1**), dihydrobotrydial (**2**), botryendial (**3**), botryenalol (**4**), β-O-methyldihydrobotrydialone (**5**), botrylactone (**6**), botcinic acid (**7**), 3-acetylbotcinic acid (**8**), botcinin A (**9**) (Dalmais et. al., 2011).

1.3.2. Blackleg disease

L. maculans is one of the most important pathogens of *Brassica* crops, including oilseed rape, rutabaga, and turnip, causing stem canker or blackleg disease

(Pedras, 1998). Annual canola crop losses caused by blackleg are estimated to be nearly 50 million Canadian dollars (Beatty and Jensen, 2002). Isolates of *L. maculans* from oilseed rape and cabbage have been identified as either aggressive or weakly aggressive. The weakly aggressive isolates have been reclassified as *L. biglobosa*. Isolates of *L. maculans* can cause all the kinds of symptoms on oilseed rape (*B. rapa*), whereas isolates of *L. biglobosa* cause necrotic lesions on leaves and cause superficial stem infection only after wounding, after insect or herbicide damage, or if the host plant is close to maturity.

Ferezou et al. (1977) first described the production of the toxin sirodesmin PL (**10**) and deactylsirodesmin PL (**11**) in liquid cultures of *L. maculans*. Pedras et al. later reported the occurrence of other sirodesmin PL (**10**) related toxins: sirodesmin H (**12**), J (**13**) and K (**14**) (Pedras et al., 1988; 1989) (Figure 1.2). Phomamide (**15**) was isolated from *L. maculans* but did not show any phytotoxic activity (Pedras, 2001). Although the blackleg fungus is a host-selective pathogen, sirodesmins are non host-specific, as they cause necrosis and cell death of both host and non-host plants. Phomalide (**16**) is a host-selective toxin isolated from 30 to 60-hour-old cultures of blackleg fungus (Pedras and Bisenthal, 1998; Pedras et al., 1993). It has been reported that *L. maculans* isolates Laird 2 and Mayfair 2 are unique because of the production of unusual secondary metabolites (in MM). Both Laird 2 and Mayfair 2 produce polanrazines, a group of sesquiterpenes, and a unique depsipeptide named depsilairdin (**18**) (Pedras et al. 2007). Depsilairdin (**18**), produced by isolate Laird 2, was found to cause strong necrotic lesions only on brown mustard leaves (Pedras et al., 2004). Interestingly, maculansin A (**17**), a phytotoxin isolated from cultures of *L. maculans* virulent on canola (*B. napus* L. cv. Wester), was found to be more toxic to the resistant plants (*B. juncea* L. cv. Cutlass, brown mustard) than to susceptible plants (canola) (Pedras and Yu, 2008).

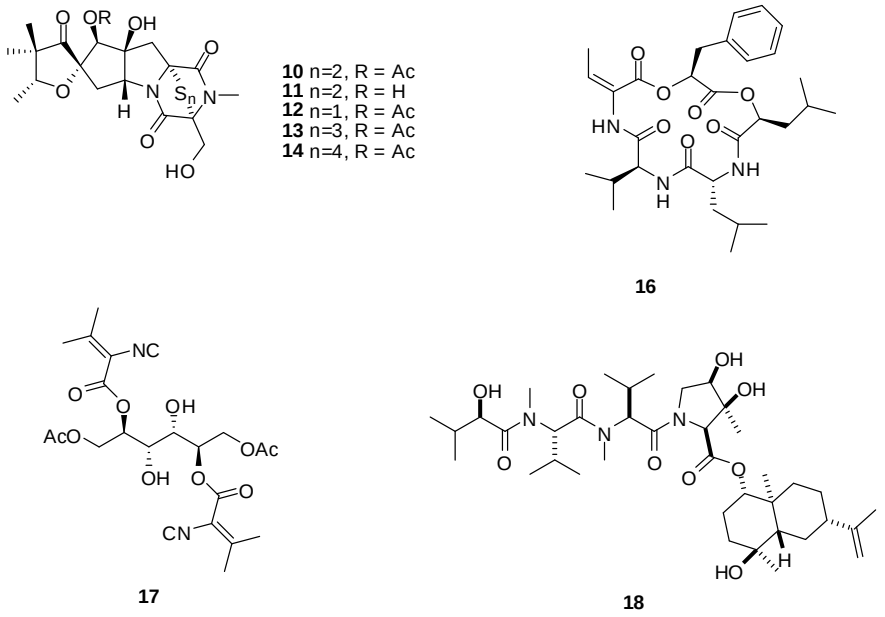
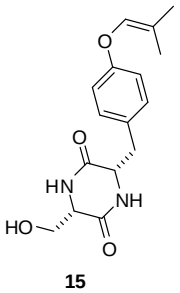


Figure 1.2 Structures of phytotoxins from *Leptosphaeria maculans*: sirodesmin PL (**10**), deacetylsirodesmin PL (**11**), sirodesmin H (**12**), sirodesmin J (**13**), sirodesmin K (**14**), phomalide (**16**), maculansin A (**17**), depsilairdin (**18**) (Pedras and Yu, 2008).

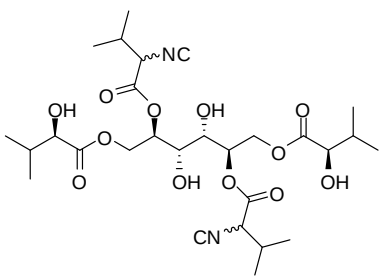


Phomamide (**15**)

1.3.3. Alternaria black spot disease

A. brassicicola together with *A. brassicae*, cause one of the most economically important diseases of *Brassica* species, Alternaria black spot (also called dark leaf spot) (Verma and Saharan, 1994). The yield loss due to black spot is very significant; up to

30% yield losses was recorded in heavily infected canola crops in Canada (Rimmer et al., 2007). Brassicicolin A (**19**) was the first metabolite reported and isolated from cultures of *A. brassicicola* (Ciegler and Lindenfelser, 1969), but its potential phytotoxic effects on brassicas was not determined. The structure of brassicicolin A (**19**) was established as a mixture of epimers by Gloer and co-workers (Gloer et al., 1988). Later Pedras and co-workers isolated brassicicolin A (**19**) from liquid cultures of *A. brassicicola* and the phytotoxic activity was determined on leaves of *B. juncea* cv. Cutlass (susceptible), *B. napus* cv. Westar (tolerant), and *Sinapis alba* cv. Ochre (resistant) (Pedras et al., 2009a). Results of phytotoxicity indicated that brassicicolin A (**19**) is a host-selective phytotoxin, causing chlorosis and necrosis on leaves of susceptible species (*B. juncea*), but not on resistant species leaves. The major phytotoxic compounds produced by *A. brassicae* have been characterized chemically and consist of destruxin B (**20**) and related compounds homodestruxin (**21**) and desmethyldestruxin (**22**) (Ayer and Pena-Rodriguez, 1987) (Figure 1.3). Destruxin B (**20**) causes chlorotic and necrotic foliar lesions on diverse *Brassica* species and other cruciferous host-plants (Pedras and Smith, 1997). *A. brassicicola* was also reported to produce a host-specific toxin protein called AB-toxin (Otani et al., 1998), from spores germinating on host leaves. In addition, several fusicoccane-like diterpenes were reported from *A. brassicicola* (MacKinnon et al., 1999; Pedras et al., 2009a).



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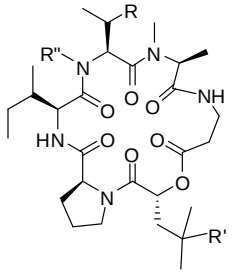

20 R = Me; R' = H; R" = Me
21 R = Et; R' = H; R" = Me
22 R = Me; R' = R" = H

Figure 1.3 Structures of host selective phytotoxins from *Alternaria brassicicola* and *Alternaria brassicae*: brassicicoline A (**19**), destruxin B (**20**), homodestruxin B (**21**), desmethyldestruxin B (**22**) (Pedras et. al., 2009a).

1.3.4. Root rot disease

Damping off and root rot diseases caused by *R. solani* are widespread in western Canada especially in the northern prairies and are usually caused by anastomosis group AG 2-1 (Sippell et al., 1985). Pre- and post-emergence damping-off of oilseed rape (*B. napus*) and canola is common wherever the crop is grown. The disease is particularly prominent in western Canada, with varying severity throughout the other regions. In Alberta, root rot has become a serious disease problem with crop infection reaching 80 - 100% of total plant numbers, resulting in partial or almost complete loss of plant stands (Kataria and Verma, 1992). Metabolites isolated from culture filtrate extracts of *R. solani* are phenyl acetic acid (**23**), *m*-hydroxyphenylacetic acid (**24**) and *p*-hydroxyphenylacetic acid (**25**) (Figure 1.4), which were considered to be phytotoxins due to their toxicity to roots of sugar beet (Nishimura and Sasaki, 1963; Frank and Francis, 1976). These toxins had similar effects on radish, beet, and corn seedlings and acted as growth hormones in low concentration.

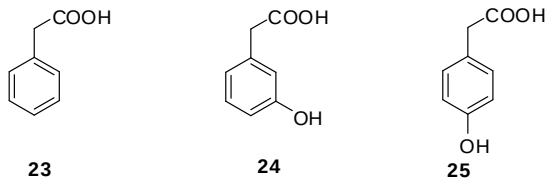

Figure 1.4 Phytotoxins isolated from root rot fungus *Rhizoctonia solani*: phenylacetic acid (**23**), *m*-hydroxyphenylacetic acid (**24**), *p*-hydroxyphenylacetic acid (**25**).

1.3.5. Stem rot disease

Sclerotinia stem rot or cottony soft rot is caused by *S. sclerotiorum* and is common around the world in the temperate regions (Boland and Hall, 1994). The disease is also serious during storage of cruciferous vegetables. *S. sclerotiorum* has a very broad host range consisting of 42 subspecies or varieties, 408 species, 278 genera, and 75 families of plants. This includes 48 members of Brassicaceae (Boland and Hall, 1994). Up to 50% yield losses were reported due to Sclerotinia stem rot in canola and rapeseed (*B. napus*, *B. rapa*), depending on environmental and weather conditions (Pedras and Ahiahonu, 2004; Lefol et al., 1997).

Oxalic acid (**26**) was reported as pathogenicity factor for *S. sclerotiorum* (Godoy et al., 1990). The role of oxalic acid (**26**) in the pathogenicity of *S. sclerotiorum* has been confirmed by using *A. thaliana* (L.) as a model system. Mutants of *S. sclerotiorum* deficient in oxalic acid (**26**) were not pathogenic to *A. thaliana* but their natural isolates were (Dickman and Mitra, 1992). Although oxalic acid (**26**) was reported to cause wilt damage to sunflower (*Helianthus annuus* L.) and other plant species (Hu et al., 2003), it did not cause any macroscopic damage to *B. napus*, *B. juncea*, *Sinapis alba*, and *Erucastrum gallicum* (Willdenow) O.E. Schulz (Pedras and Ahiahonu, 2004). However, it has been reported that *S. sclerotiorum* produces sclerin

(**27**) that is phytotoxic to three cruciferous species (*B. napus*, *B. juncea*, *S. alba*) susceptible to *Sclerotinia* stem rot disease, but not to resistant species (*E. gallicum*) (Pedras and Ahiahonu, 2004). Several other polyketides such as sclerolide (**28**), sclerotinin (**29**), sclerotinin B (**30**), sclerone (**31**) and isosclerone (**32**) have been isolated from *S. sclerotiorum*, although their phytotoxic activity has not been determined (Figure 1.5).

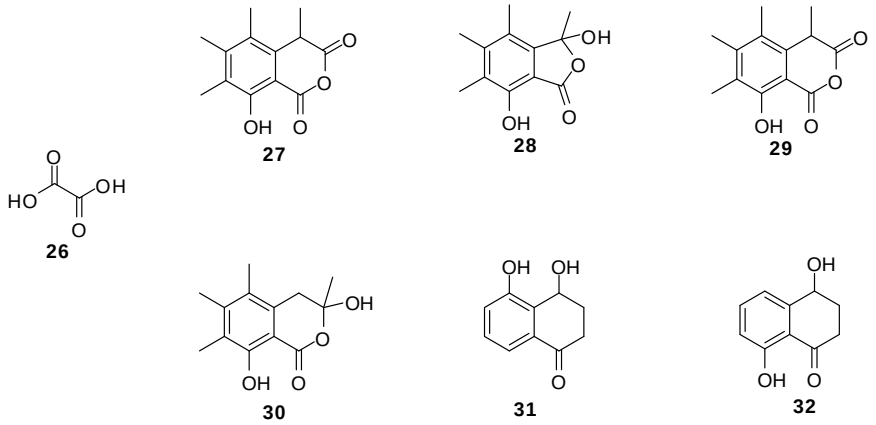

Figure 1.5 Secondary metabolites isolated from liquid cultures of *Sclerotinia sclerotiorum:* oxalic acid (**26**), sclerin (**27**), sclerolide (**28**), sclerotinin A (**29**), sclerotinin B (**30**), sclerone (**31**), isosclerone (**32**).

1.4. Crucifereous chemical defenses

Cruciferous chemical defenses include two major groups of compounds: phytoalexins and phytoanticipins. Both phytoalexins and phytoanticipins have been reported to play crucial roles in cruciferous defense against fungal pathogens and insects (Pedras et al. 2011a).

1.4.1. Cruciferous phytoalexins

The cruciferous phytoalexins have been reviewed recently (Pedras et al., 2011a), for this reason only those pertinent to work described in this thesis are reviewed. Phytoalexins are induced in both wild and crop crucifers because of biotic and abiotic stresses (Pedras et al., 2000). Brassinin (**33**), 1-methoxybrassinin (**34**) and cyclobrassinin (**35**) are the first reported cruciferous phytoalexins, isolated from Chinese cabbage (Takasugi et al., 1986). The phytoalexins of crucifers are indole alkaloids derived from (*S*)-tryptophan (**42**). Until now, 44 cruciferous phytoalexins have been reported from cruciferous plants (Pedras et al., 2011a). All the phytoalexins isolated from cruciferous plants share a common core structure with an indole or related ring. Pedras et al. (2011) categorized cruciferous phytoalexins in six different groups according to the structural similarities (Figure 1.6). Group A compounds contain an indole or 2-oxoindole and a nitrogen atom at C-1´ (e.g. brassinin (**33**)); group B compounds contain a ring fused to indole (e.g. cyclobrassinin (**35**)); group C, (*S*)-dioxibrassinin (**37**) and structures containing a spirocyclic ring (e.g. spirobrassinin (**36**)); group D compounds contain an indole and an aldehyde or ester substituent at C-3 (e.g. brassicanal A (**38**)); group E compounds contain an indole and a thiazole substituent at C-3 (e.g. camalexin (**39**)); group F compounds contain an indole and an ethylnitryl substituent at C-3 (e.g. indolyl-3-acetonitrile (**40**)); and group G compounds contain the phytoalexins that do not fit into any of the groups A- F (e.g. brussalexin A (**41**)) (Pedras et al., 2011a).

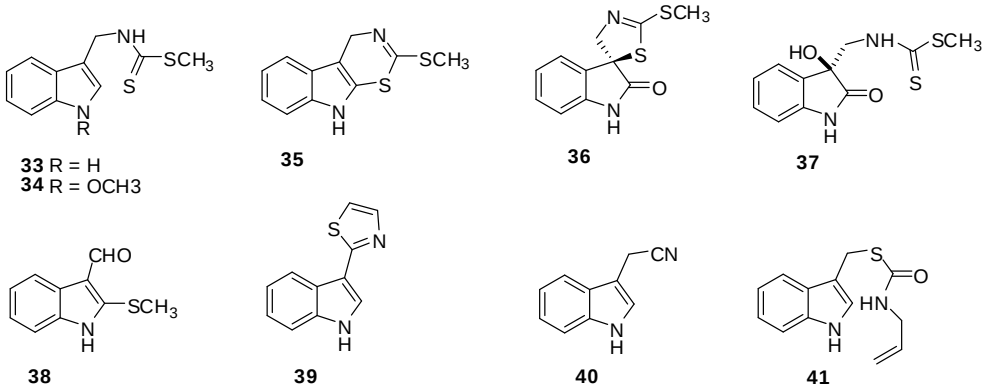

Figure 1.6 Examples of phytoalexins produced in cruciferous plants: brassinin (**33**), 1-methoxybrassinin (**34**), cyclobrassinin (**35**), spirobrassinin (**36**), dioxibrassinin (**37**), brassicanal A (**38**), camalexin (**39**), indole-3-acetonitrile (**40**), brussalexin A (**41**).

Brassinin (**33**) and camalexin (**39**) are two very important phytoalexins of crucifers. Brassinin (**33**) is produced in cultivated species, but so far, camalexin (**39**) has been reported only in wild crucifers (Pedras et al., 2011a). Both compounds showed high antifungal activity against many cruciferous fungal pathogens. Both brassinin (**33**) and camalexin (**39**) are derived from (*S*)-tryptophan (**42**) via indolyl-3-acetaldoxime (**43**). The biosynthetic pathway of camalexin (**39**) was found to be different from that of brassinin (**33**) as indolyl-3-acetonitrile (**40**), derived from indolyl-3-acetaldoxime (**43**), is an intermediate in the biosynthesis of camalexin (**39**) but not brassinin (**33**) (Figure 1.7) (Pedras et al., 2011a).

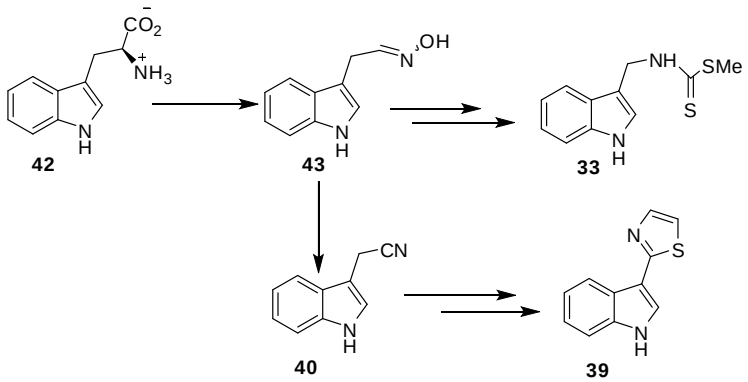

Figure 1.7 Biosynthesis of brassinin (**33**) and camalexin (**39**).

The phytoalexin brassinin (**33**) is also an intermediate of many other phytoalexins such as cyclobrassinin (**35**), spirobrassinin (**36**), brassicanal A (**38**), brassicanate A (**44**) and cyclobrassinin (**35**), which in turn is an intermediate between brassinin (**33**), brassilexin (**45**) and rutalexin (**46**) (Figure 1.8). Similar to brassinin (**33**), 1-methoxybrassinin (**34**) has been reported as an intermediate to several other phytoalexins (Pedras et al., 2011a).

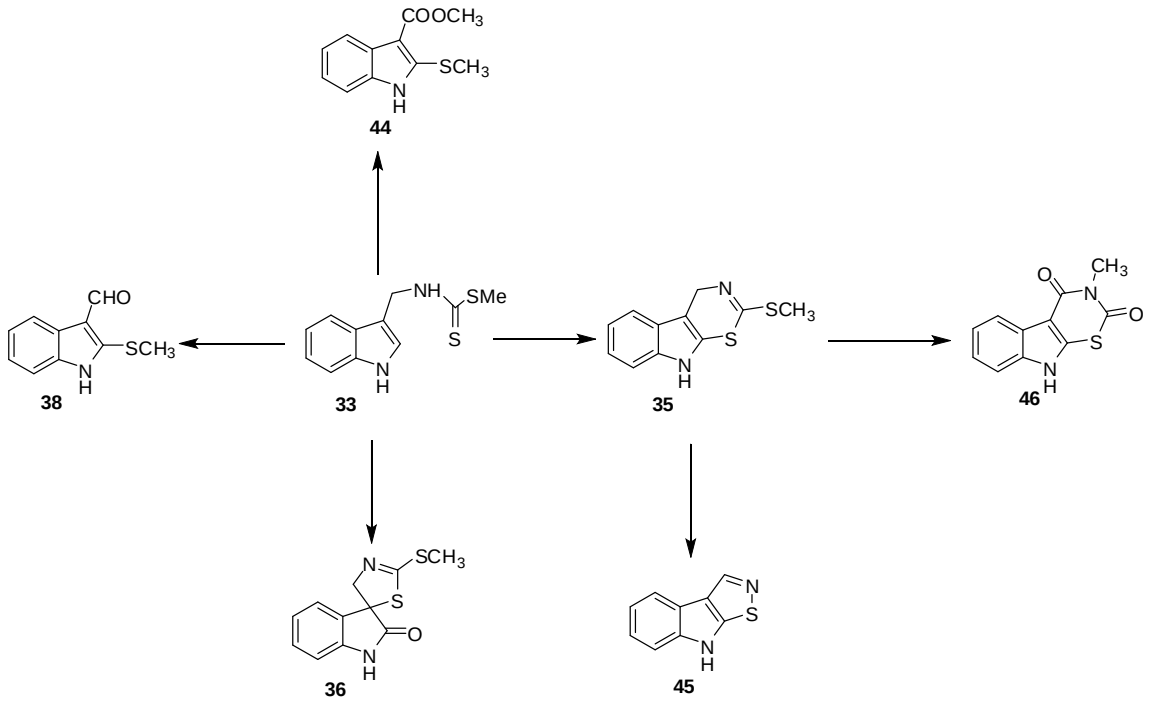

Figure 1.8 Biosynthetic relationship between brassinin (**33**) and other phytoalexins (Pedras et al., 2011a).

Metabolic detoxification of phytoalexins is one of the ways that pathogens overcome plant defenses. Several fungal pathogens of crucifers have been reported to produce enzymes that can detoxify important phytoalexins of crucifers. For example *L. maculans* oxidized brassinin (**33**) to indole-3-carboxaldehyde (**47**) and indole-3-carboxylic acid (**48**) whereas, *L. biglobosa* and *A. brassicicola* hydrolyzed brassinin (**33**) to indolyl-3-methanamine (**49**) and $N_{\mathbf{b}}$-acetyl-3-indolylmethanamine (**50**). On the other hand, *S. sclerotiorum* glucosylated brassinin (**33**) to 1-β-D-glucopyranosylbrassinin (**51**) (Figure 1.9) (Pedras and Ahiahonu, 2005; Pedras and Ahiahonu, 2002; Pedras and Khan, 2000; Pedras et al. 2011a).

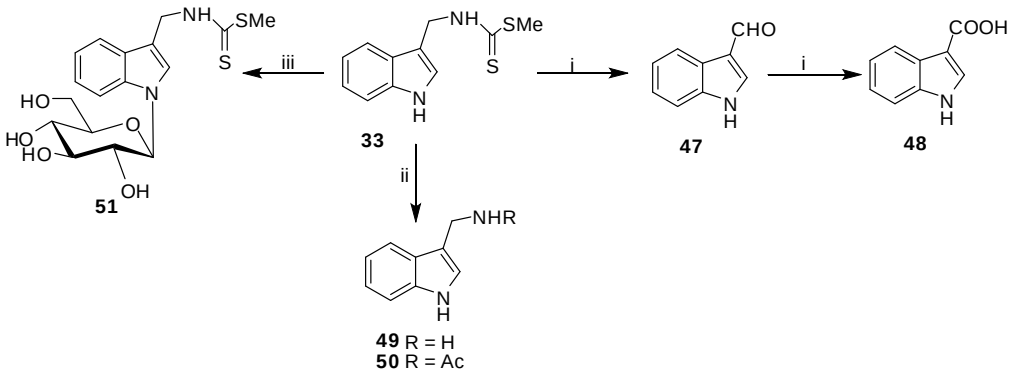

Figure 1.9 Metabolism of brassinin (**33**) (i) by *Leptosphaeria maculans*; (ii) *Leptosphaeria biglobosa* and *Alternaria brassicicola* and (iii) *Sclerotinia sclerotiorum*

Camalexin (**39**) was detoxified by *R. solani* to two metabolites **53** and **54** via 5-hydroxycamalexin (**52**) (Pedras and Khan, 2000) and to 6-oxy-(O-β-D-glucopyranosyl)camalexin (**56**) via 6-hydroxycamalexin (**55**) by *S. sclerotiorum* (Figure 1.10) (Pedras and Ahiahonu, 2002).

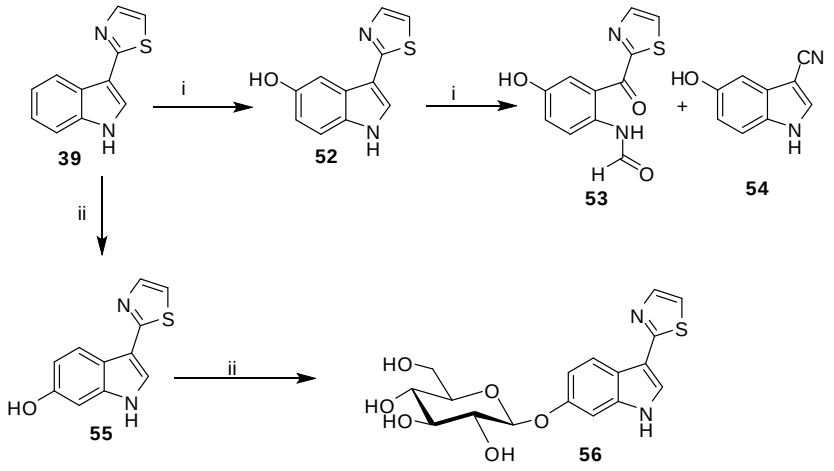

Figure 1.10 Metabolism of camalexin (**39**) by (i) *Rhizoctonia solani* and (ii) *Sclerotinina sclerotiorum.*

It has been reported that several other phytoalexins (**34**, **35**, **45**, and **57**) were also metabolized by *S. sclerotiorum* via glucosylation (Figure 1.11) (Pedras et al., 2011a).

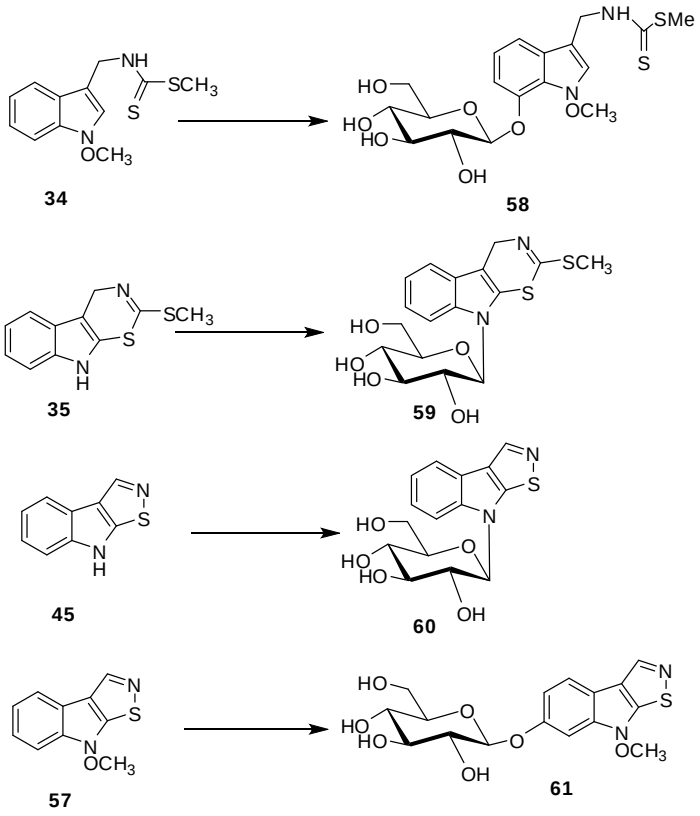

Figure 1.11 Metabolism of 1-methoxybrassinin (**34**), cyclobrassinin (**35**), brassilexin (**45**), and sinalexin (**57**) by *Sclerotinia sclerotiorum.*

The purification and characterization of brassinin hydrolases produced by *L. maculans* (BHLmL2) and *A. brassicicola* (BHAb) were accomplished by Pedras and co-workers (Pedras et al., 2009b). Native BHLmL2 was found to be a tetrameric protein with a molecular mass of 220 kDa, whereas native BHAb was found to be dimeric protein of 120 kDa. Protein characterization by LC-MS/MS and sequence analysis suggested that both enzymes belong to the family of amidases with the

catalytic Ser/Ser/Lys triad. The substrate specificity of the purified enzymes (BHLmL2 and BHAb) was tested against various synthetic compounds containing a dithiocarbamate group or isosteres of brassinin containing indolyl-3-methyl or napthyl-1 or napthyl-2 moieties. Results have shown that both the BHs are functional group specific and brassinin (**33**) is the best substrate among all the compounds tested (Pedras et al. 2009b; Pedras et al., 2012).

Brassinin oxidase (BO) from *L. maculans* was purified and characterized by Pedras and co-workers (2008). Purified brassinin oxidase, which is approximately 20% glucosylated, has an apparent molecular mass of 57 kDa. The enzyme BO accepts a wide range of cofactors, including quinones and flavins (Pedras et al., 2008). To find inhibitors of the enzyme 78 compounds containing cruciferous phytoalexins and analogues were tested for inhibition, but only four compounds showed activity. Two phytoalexins camalexin (**39**) and cyclobrassinin (**35**) are competitive inhibitors of BO (Pedras et al., 2008). These were the first molecules reported as inhibitors for a phytoalexin detoxifying enzyme. None of the compounds was transformed by BO, which indicated that BO is substrate specific. Protein characterization by LC-MS/MS and sequence analysis of BO did not show a significant match with proteins available in the NCBI database (Pedras et al., 2008).

The gene (*SsBGT1*) responsible for transformation of brassinin (**33**) by *S. sclerotiorum* was cloned and expressed in *Saccharomyces cerevisiae* and the enzyme (SsBGT1) was purified and characterized by Sexton et al. (Sexton et al., 2009). The purified enzyme, SsBGT1 showed lower glucosylation activity on cyclobrassinin (**35**) than cell-free extracts of mycelia from phytoalexin-induced cultures of *S. sclerotiorum* (5% vs. 83%). Similarly, brassilexin (**45**) was not a substrate of SsBGT1 though in cell cultures brassilexin (**45**) was glucosylated by *S. sclerotiorum*. Alignment of the amino

acid sequence of SsBGT1 with glucosyltransferases from other fungal species showed the closest match in *B. cinerea* with a 57% amino acid identity (Sexton et al., 2009). This sequence similarity between *S. sclerotiorum* and *B. cinerea* is not surprising considering the phylogenetic relationship between these two species, as both are belong to the family Sclerotiniaceae (Hirschhaeuser and Froehlich, 2007).

1.4.2. Cruciferous phytoanticipins

Glucosinolates are phytoanticipins produced in Brassicaceae and fifteen other plant families, and are partly responsible for the flavor and other properties of these vegetables (Fahey et al., 2001). The skeleton of glucosinolates consists of a thioglucosidic bond to the carbon of a sulfonated oxime with a variable side chain (Figure 1.12). The side chain is derived from different amino acids; it can be aliphatic (e.g. alkyl, alkenyl, hydroxyalkenyl), aromatic (e.g. benzyl, substituted benzyl) or heterocyclic (e.g. indolyl). Since the isolation of the first two glucosinolates sinigrin (**131**) and sinalbin (**72**) from black mustard (*B. nigra*) and white mustard (*S. alba*) seeds respectively in 1980s more than 120 glucosinolates were reported (Fahey et al., 2001; Mithen et al., 2010). Most of the glucosinolates produced in plants are aliphatic; aromatic and indolyl glucosinolates produced in crucifers are listed in figure 1.13 and figure 1.14 (Fahey et al., 2001; Halkier and Gershenzon, 2006). Although glucosinolates have been reported almost exclusively from the order of Capparales, which contain 15 families including Brassicaceae (crucifers), some glucosinolates have been isolated from the genus *Drypetes* from the family Euphorbiaceae that is completely unrelated to other glucosinolate containing families (Halkier and Gershenzon, 2006; Mithen et. al., 2010).

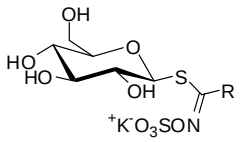

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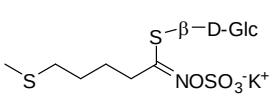
Figure 1.12 General structure of glucosinolates

The distribution of glucosinolates and their concentrations are species dependent as well as plant age and tissue dependent. Not only total glucosinolate levels, but also the glucosinolate composition may vary significantly among different tissues of the same plant (Redovnikovic, et al., 2008). Accumulation of glucosinolates in the Columbia ecotype of *A. thaliana* at different stages of its life cycle was studied by Gershenzon and co-workers, who reported significant differences among organs in concentration and composition (Brown et al., 2003). The highest concentration was found in reproductive organs such as flowers and seeds, followed by young leaves, while senescing rosette leaves contained the lowest concentration of glucosinolates. Intermediate concentration was found in leaves, stems, and roots. In seedlings, the cotyledons had the highest glucosinolate levels, whereas in vegetative plants, roots overall had higher glucosinolate levels than leaves, and younger leaves have higher glucosinolate content than older leaves. The main glucosinolates of seeds, 4-methylthiobutylglucosinolate (**63**), and 4-benzyloxybutylglucosinolate (**83**) are only minor in leaf and root tissues, whereas 4-methylsulfinylbutylglucosinolate (**64**) is the major glucosinolate (Brown et al., 2003). Both qualitative and quantitative variation in the composition of 34 different glucosinolates has been observed in leaves and seeds of 39 ecotypes of *A. thaliana* (Kliebenstein et al., 2001). In *Brassica* species 2-phenylethylglucosinolate (**67**) was found in higher quantity in roots than in shoots (35% vs. 6%) (van Dam et al., 2005).



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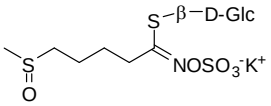

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Glucosinolates produced by four Brassica species (*B. nigra, B. carinata, B. juncea,* and *B. rapa*) were investigated by Sørensen and co-workers (Bellostas et al., 2004) using plants at four different development stages. Total glucosinolate levels were found up to 120 µmol/g of dry mass in *B. nigra* and *B. juncea*, while *B. rapa* produced maximum 25 µmol/g of dry mass at any stage of the plant life (Bellostas et al., 2004). In *B. nigra*, *B. carinata*, and *B. juncea* reproductive tissues showed the highest glucosinolate concentration when compared to the rest of the plant parts, while in *B. rapa*, the roots produced highest glucosinolate amounts compared to other parts of the plant. Aliphatic glucosinolates were mainly present in vegetative parts of the species, accounting for approximately 50% of the glucosinolate content of the roots in all species except *B. rapa*. Phenylethylglucosinolate (**67**) was the only aromatic glucosinolate present in all species and was the dominant compound in roots of *B. rapa* and 50% of root glucosinolates in *B. juncea*. Glucobrassicin (**86**) was present in higher proportion in *B. rapa* compared to other species, especially in roots and reproductive tissues (Bellostas et al., 2004).

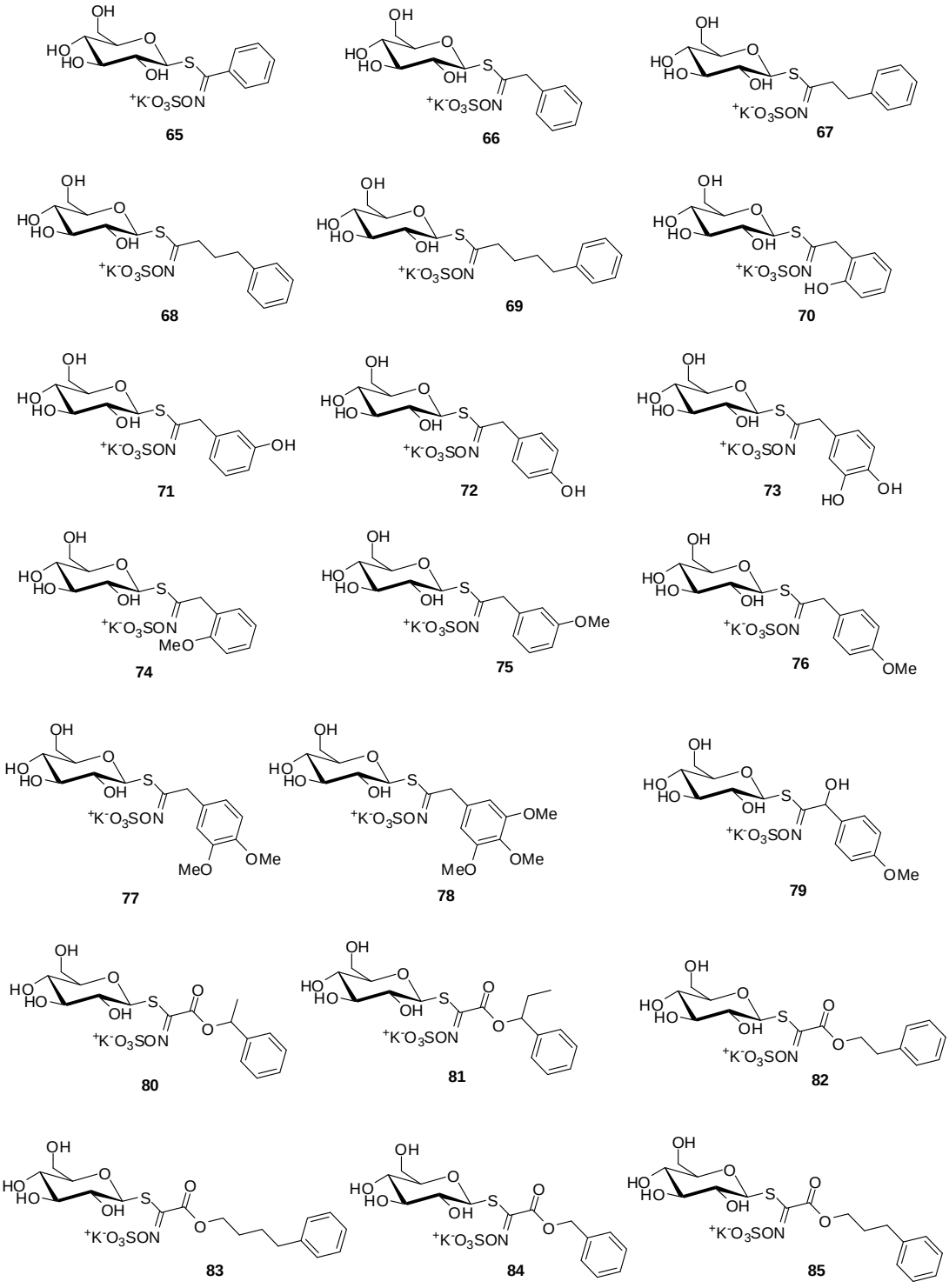


Figure 1.13 Aromatic glucosinolates produced in cruciferous plants (Fahey et al., 2001; Mithen et al., 2010).

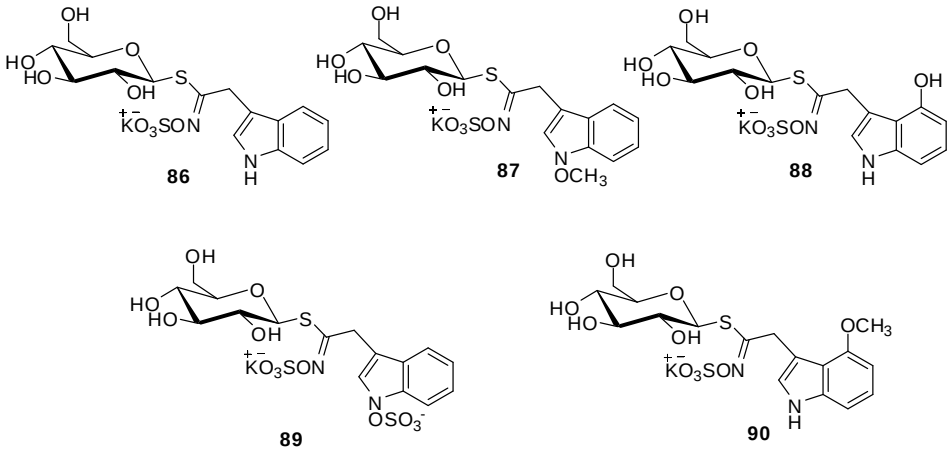


Figure 1.14 Indolyl glucosinolates produced in cruciferous plants (Fahey et al., 2001; Mithen et al., 2010).

Glucosinolates co-occur with myrosinases, thioglucosidases (EC 3.2.1.147) from plants stored in separate organelles. Glucosinolates are found in vacuoles whereas myrosinases are located in myrosin cells scattered in plant tissues and found especially in phloem, parenchyma and stomata cells (Ahuja et al., 2010; Bones and Rossiter, 2006; Kissen et al., 2009).

Upon tissue damage glucosinolates (**62**) are exposed to myrosinases and are hydrolyzed to yield an aglycon (**91**), which undergoes rearrangement to produce the corresponding isothiocyanates (**92**) by a Lossen-type rearrangement, thiocyanates (**94**), and nitriles (**93).** Other products are also formed depending on pH, metal ions and proteins produced in plants and nature of the side chain (Figure 1.15) (Bones and Rossiter, 2006). Formation of nitriles is an enzymatic process using ESP (epithiospecifier protein), NSP (nitrile specifier protein) and Fe^{+2}. Many plant species possess additional proteins named specifier proteins, such as epithiospecifier proteins promotes the formation of epithionitriles (Bones and Rossiter, 2006; Kissen and Bones, 2009).

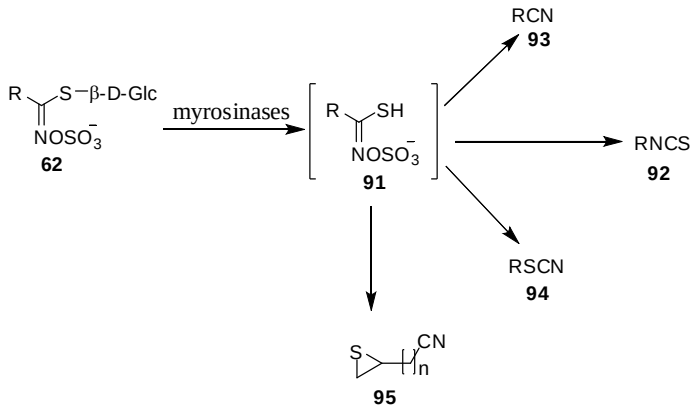


Figure 1.15 Transformation of glucosinolates (**62**) by myrosinases (Bones and Rossiter, 2006).

The isothiocyanates (**99-101**) of indole glucosinolates (**86**, **87**, and **90**) are not stable, decomposing to indolyl-3-methylene carbocations (**104-106**). In the plant, these carbocations react with nucleophiles such as water, ascorbate, glutathione, etc., to yield indolyl-3-methanols (**110-112**) and ascorbigens (**107-109**) (Figure 1.16) (Agerbirk et al., 2009; Bones and Rossiter, 2006). In addition, from the thiohydroximate intermediates (**96-98**), indolyl-3-acetonitriles (**40**, **102**, and **103**) are formed and indolyl-3-methylenes (**104-106**) react with indolyl-3-methanols (**110-112**) to form diindolyl-3-methanes (**113-115**) (Figure 1.16).

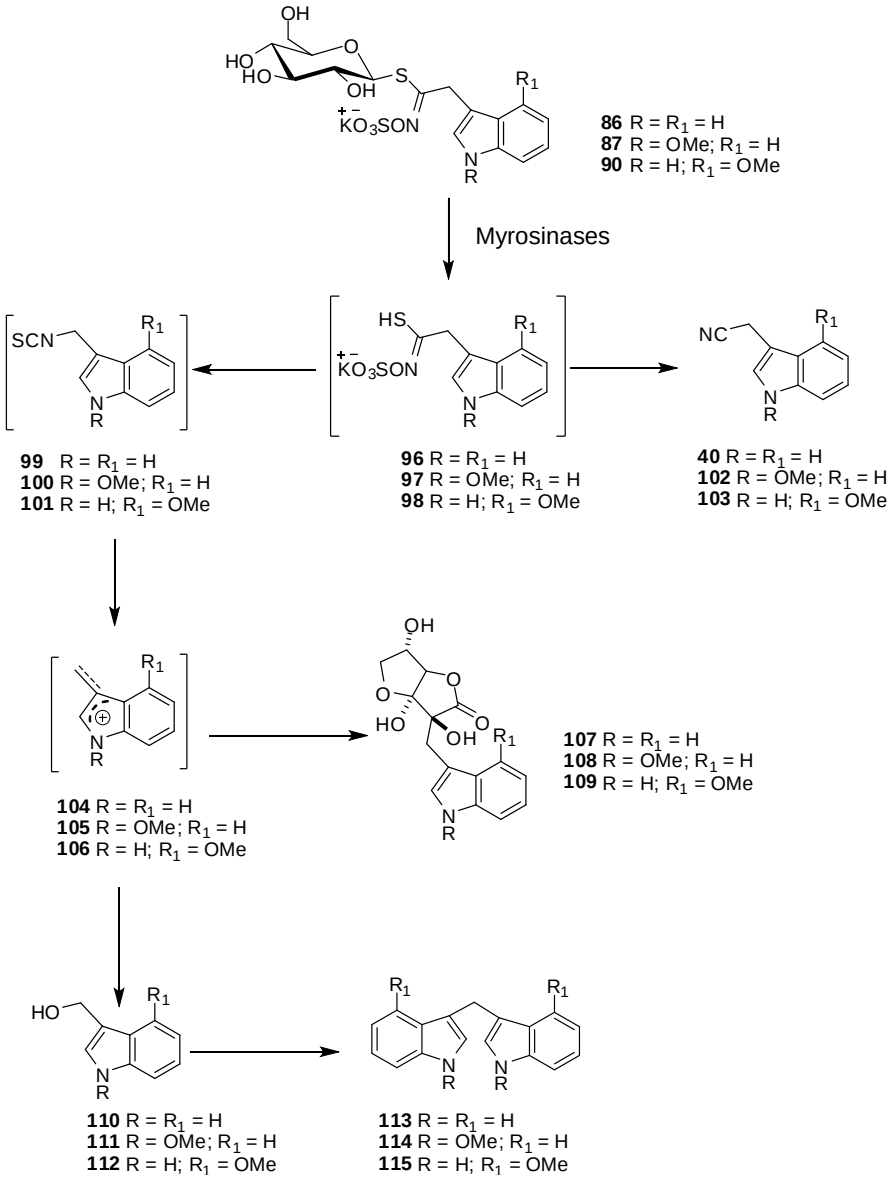


Figure 1.16 Transformation of indolylglucosinolates (**86**, **87**, and **90**) by myrosinases and subsequent transformation in plant tissues (Bones and Rossiter, 2006).

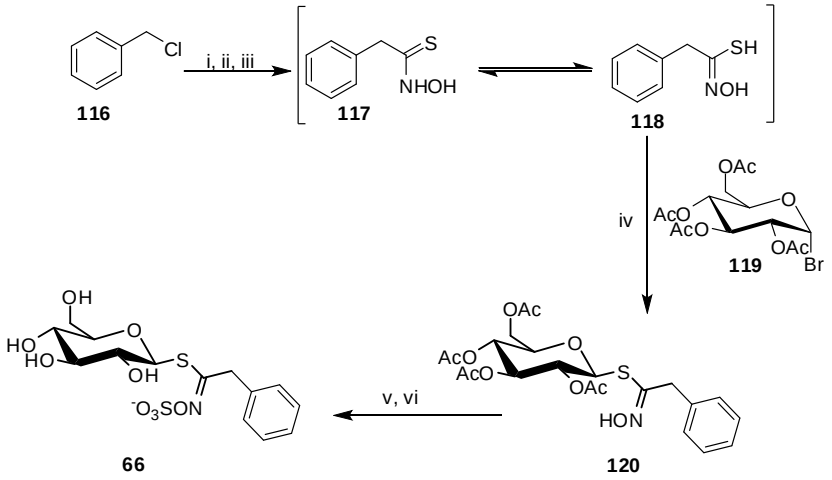
Synthesis and biosynthesis of glucosinolates

The synthesis of glucosinolates was recently reviewed by Rollin and Tatibouët (Rollin and Tatibouët, 2011). The biosynthesis of glucosinolates have been reviewed

extensively by several authors (Wittstock and Halkier, 2002; Mikkelsen et al., 2002; Falk et al., 2004; Halkier and Gershenzon, 2006; Grubb and Abel, 2006; Sønderby et al., 2010).

Synthesis of glucosinolates

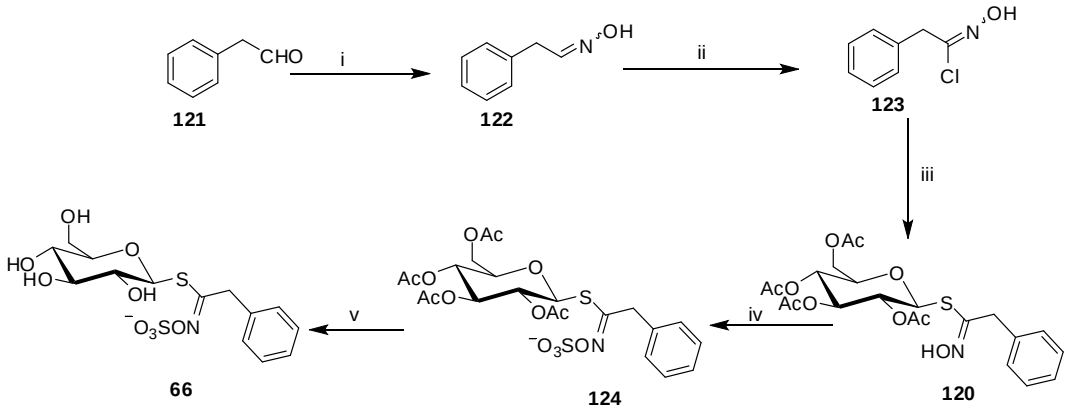
The first synthesis of an aryl glucosinolate was reported by Ettlinger and Lundeen (1957) where they described the synthesis of benzylglucosinolate (**66**) through phenylacethiohydroxamic acid (**118**) starting from benzyl chloride (**116**) with an overall yield of 7% (Scheme 1.1). This synthetic route afforded a low overall yield, which is mostly due to the low yield and stability of thiohydroxamic acid **118**.



Scheme 1.1 Synthesis of benzyl glucosinolate (**66**). Reagents: (i) Mg, Et_2O; (ii) CS_2; (iii) $NH_2OH.HCl$; (iv) KOH, CH_3OH: acetone (1:3); (v) $Pyr.SO_3$.pyridine; (vi) MeOH, NH_3 (Ettlinger and Lundeen, 1957).

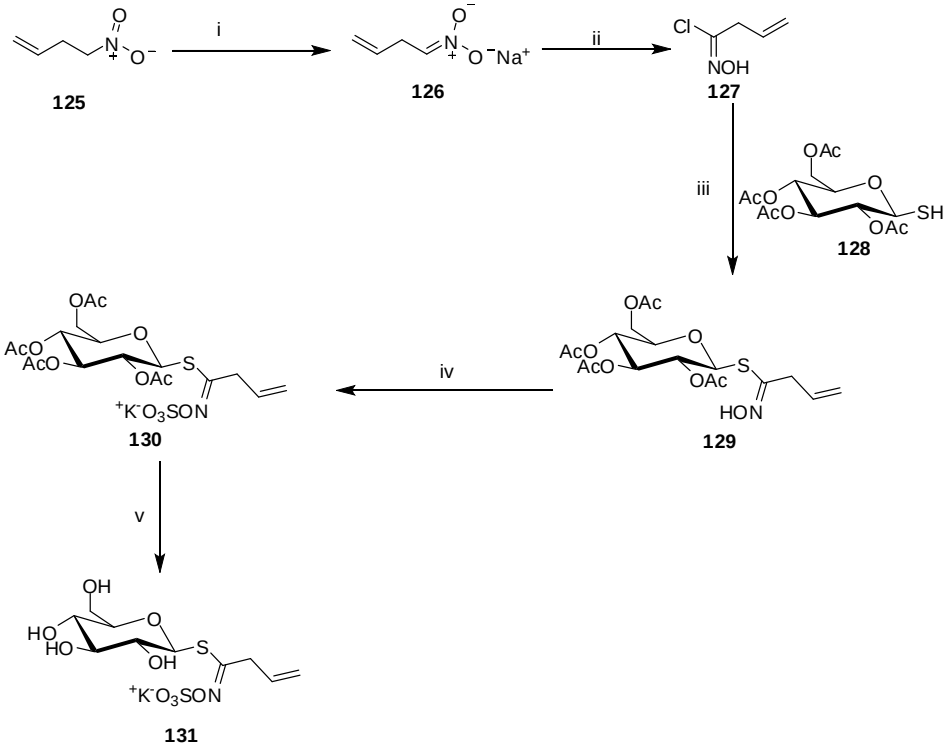
An alternative synthetic route was reported by Benn (1964) for aliphatic and aromatic glucosinolates from aldoxime. Chlorination of aldoximes using chlorine gas

afforded the corresponding hydroximoyl chlorides, which were then used as crude intermediates to couple with tetra-O-acetylated thioglucopyranoses to yield the thiohydroximates. Subsequent O-sulfation with sulfur trioxide pyridine complex followed by treatment with $KHCO_3$ resulted in the formation of tetraacetylated glucosinolate, which was then hydrolyzed with a suitable base to deliver the expected glucosinolates in much better yield (30%) compared to the earlier method (Scheme 1.2).



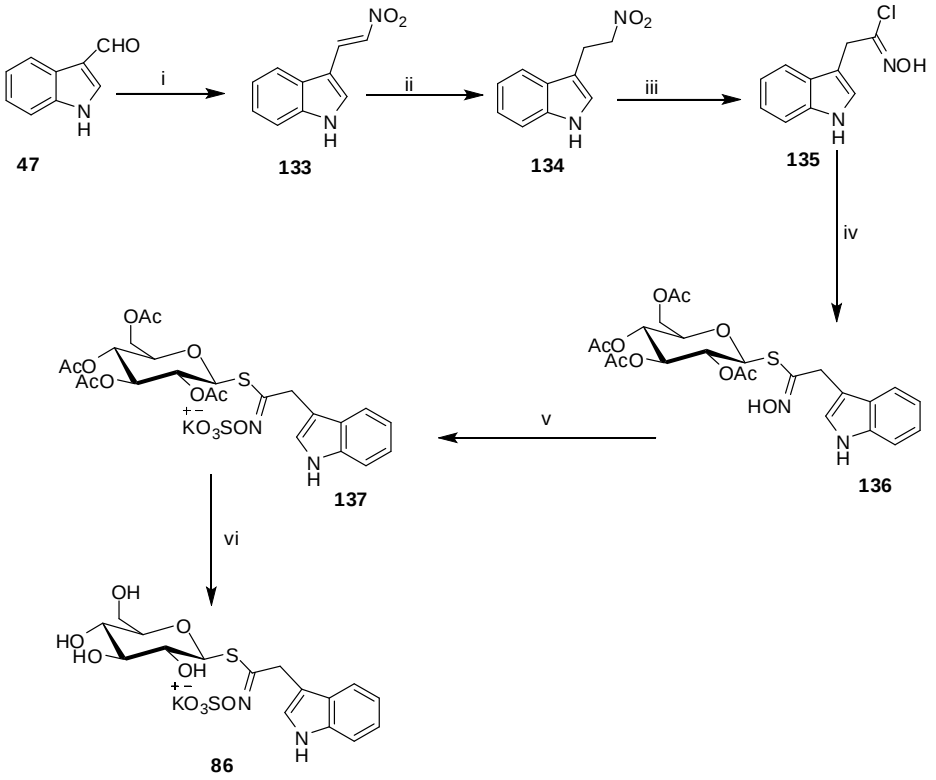
Scheme 1.2 Synthesis of benzylglucosinolate (**66**). Reagents: (i) $NH_2OH.HCl$, Na_2CO_3, H_2O, EtOH, 70%; (ii) Cl_2, DCM; (iii) Thioglucose, Et_3N, 78%; (iv) HSO_3Cl, pyridine, 55%; (v) MeOK, MeOH, 100% (Benn, 1964).

Synthesis of sinigrin (**131**) was accomplished following a route using nitronate (Benn and Ettlinger, 1965). The syntheses of alkenyl and indolyl glucosinolates were not possible from the aldoxime, as traditional chlorinating reagents did not work in presence of alkenyl and indolyl group. Benn and Ettlinger first treated a 4-nitrobut-1-ene (**125**) with a strong base (NaOEt) to form the corresponding nitronate (**126**) which was then treated with LiCl to produce the corresponding hydroximoyl chloride (**127**). The hydroximoyl chloride (**127**) was then converted to sinigrin (**131**) following the reactions reported earlier (Scheme 1.3).



Scheme 1.3 Synthesis of sinigrin (**131**): Reagents: (i) NaOEt, EtOH; (ii) LiCl, HCl; (iii) Thioglucose, Et_3N; (iv) HSO_3Cl, pyridine,; (v) MeOK, MeOH, (Benn and Ettlinger, 1965; Rollin and Tatibouët, 2011).

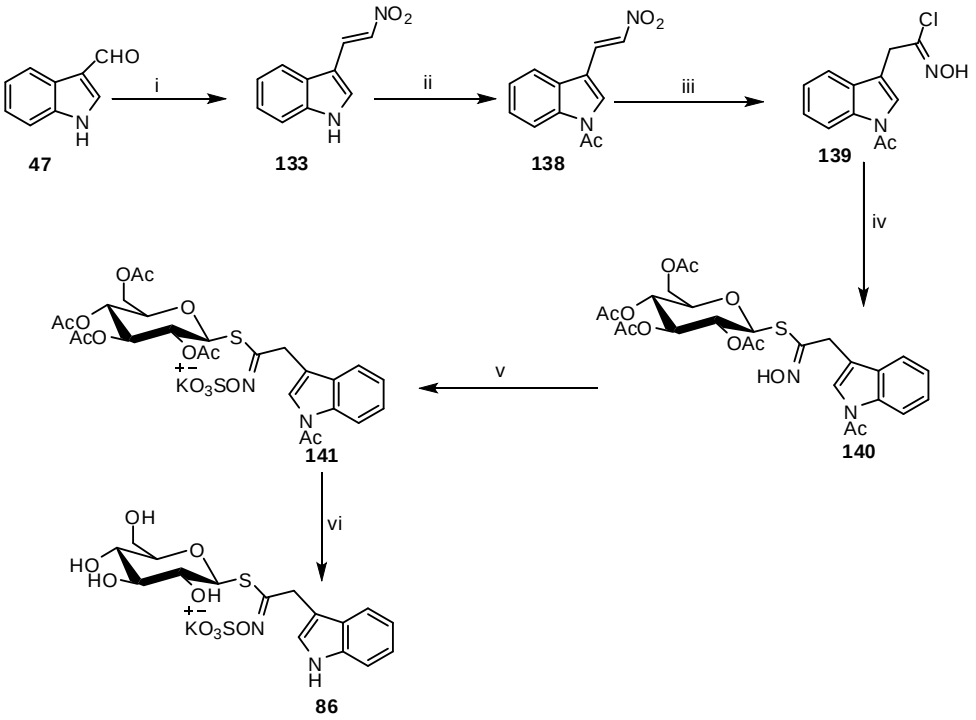
Using the procedure followed for the synthesis of sinigrin (**131**) from nitronate, Viaud and Rollin reported for the first time the synthesis of an indolyl glucosinolate (glucobrassicin, **86**) through nitronate (Viaud and Rollin, 1990). 3-Indolecarboxaldehyde (**47**) was first treated with nitromethane and ammonium acetate to give the nitrovinylindole (**133**), which was reduced with $NaBH_4$ to the nitroethylindole (**134**). Synthesis of glucobrassicin (**86**) was then accomplished from nitroethylindole (**134**) following the route reported by Benn and Ettlinger, with a slight modification (Scheme 1.4) and overall yield was 10%.



Scheme 1.4 Synthesis of glucobrassicin (**86**) Reagents: (i) CH_3NO_2, ammonium acetate, 120 -130º C; (ii) $NaBH_4$, SiO_2, $CHCl_3$, i-PrOH; (iii) MeONa, MeOH, $SOCl_2$, DME ; (iv) Thioglucose (**128**), Et_3N, Et_2O/CH_2Cl_2; (v) HSO_3Cl, pyridine; (vi) MeOK, MeOH (Viaud and Rollin, 1990).

An alternative synthetic route for the synthesis of several indolyl glucosinolates was reported by Cassel et al. from *N*-protected nitrovinylindole following Kulkarni method of one-step conversion of nitroalkenes into hydroximoyl chlorides as shown in scheme 1.5 (Cassel et. al., 1998). Although this route is relatively shorter than previous method for indolyl glucosinolates (scheme 1.4), the yield did not improve much (11% vs. 10%). The major drawback of this method is not being applicable for the synthesis

of methoxy glucobrassicins. Methoxy glucosinolates were not stable in the reaction conditions, resulted in decomposition.



Scheme 1.5 Synthesis of glucobrassicin (**86**) Reagents: (i) CH_3NO_2, ammonium acetate, 120 -130º C; (ii) Ac_2O, pyridine; (iii) $TiCl_4$, Et_3SiH, CH_2Cl_2; (iv) Thioglucose (**128**), Et_3N, Et_2O/CH_2Cl_2; (v) HSO_3Cl, pyridine, $KHCO_3$; (vi) MeOK, MeOH (Cassel et al., 1998).

Biosynthesis of glucosinolates

The biosynthesis of glucosinolates have been reviewed by several authors (Halkier and Gershenzon, 2006; Wittstock and Halkier, 2002; Mikkelsen et al., 2002; Grub and Abel, 2006). Glucosinolates are derived from protein amino acids such as alanine (Ala), leucine (Leu), isoleucine (Ile), methionine (Met), valine (Val), phenylalanine (Phe), tyrosin (Tyr), and tryptophan (Trp) (Halkier and Gershenzon,

2006; Grub and Abel, 2006). Glucosinolates derived from Ala, Leu, Ile, Met, or Val are known as aliphatic glucosinolates, those derived from Phe or Tyr are known as aromatic glucosinolates, and those derived from Trp are known as indolyl glucosinolates. The biosynthesis of glucosinolates can be divided into three stages: (i) chain elongation of certain aliphatic and aromatic amino acids via sequential addition of methylene groups, (ii) construction of the core structure of glucosinolates, and (iii) secondary modification of the side chain (Grubb and Abel, 2006; Halkier and Gershenzon, 2006; Sønderby et al., 2010).

(i) Chain elongation of amino acids

Chain elongated aliphatic glucosinolates are the most abundant glucosinolates found in crucifers, including the model plant *A. thaliana* and many other Brassicaceae species (Halkier and Gershenzon, 2006). The side chain elongation reactions have been investigated using *in vivo* labeling studies, isolation of the participating intermediates, and characterization of the enzymes catalyzing various reactions in the biosynthetic pathway (Graser et al., 2000). Side chain elongation reactions are shown in the Figure 1.17. Side chain-elongation begins with oxidative deamination of the parent amino acid (**142**) to yield the corresponding 2-oxo acid (**143**). One of the enzymes catalyzing oxidative deamination of amino acids, branched-chain aminotransferase (BCAT4) in the biosynthetic pathway of methionine-derived glucosinolates was characterized from *A. thaliana* (Schuster et al., 2006). The 2-oxo acid (**143**) enters a three step-cycle in which it (i) condensed with acetyl CoA to form a substituted 2-malate derivative (**144**) (methylthioalkylmalate synthase, MAM), followed by (ii) isomerization via a 1,2-shift of the hydroxyl group to form a 3-malate derivative (**145**) (isopropylmalate isomerase, IPMI) that is followed by (iii) oxidative decarboxylation to yield a new 2-oxo acid

(**146**) (isopropylmalate dehyrogenase, IPH-DH) (Graser et al., 2000; Halkier and Gershenzon, 2006; Falk et al., 2004; Textor et al., 2004; 2007).

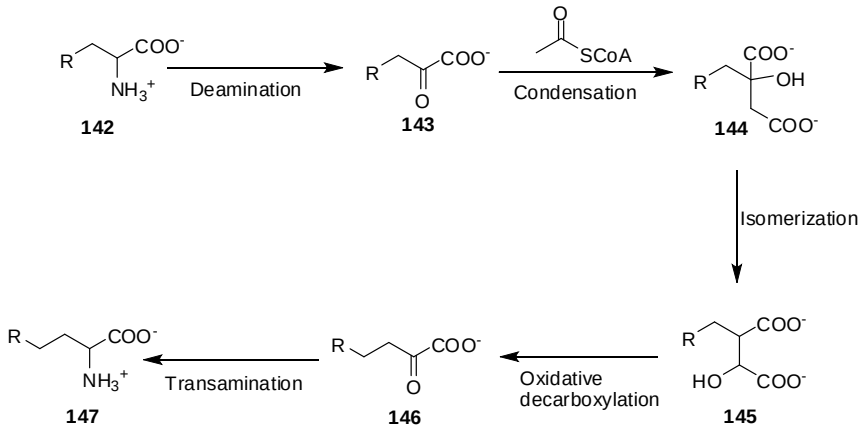


Figure 1.17 Chain elongation of aminoacids (Graser et al., 2000; Halkier and Gershenzon, 2006).

The 2-oxo acid (**146**) further transaminated to regenerate the amino acid (**147**) which enters the biosynthetic pathway of short chain aliphatic or aromatic glucosinolates, or it may undergo further cycles of chain-elongation (Textor et al., 2004; 2007). Chain elongation products of up to nine cycles are known to occur in plants (Fahey et al, 2001), but only six cycles are known in the model plant *A. thaliana* (Textor et al., 2004; 2007). All chain-elongated 2-oxo acids are converted back to the corresponding amino acids that participate in the biosynthetic pathway of glucosinolates of varying side-chain lengths (Graser et al., 2000).

Three genes *MAM1, MAM2* and *MAM3* have been identified as responsible for the condensation of acetyl-CoA in the aliphatic glucosinolate pathway. The enzyme catalyzing the first step of the chain-elongation reaction, 2-(ω-methylthioalkyl)malate

synthases (MAMS), was partially purified from rocket (Falk et al., 2004). *In vitro* experiments have shown that MAM1 is capable of catalyzing the condensation of the first three elongation cycles, MAM2 only one cycle, whereas MAM3 is capable of catalyzing all six condensation reactions methionine chain-elongation that occur *A. thaliana*. MAM3 is also exhibits a broad substrate range of other non-methionine derived 2-oxo acids (Texor et al., 2007).

(ii) Biosynthesis of the glucosinolate core structure

The biosynthesis of aliphatic, aromatic, and indolyl core glucosinolate structure involves common intermediates (*N*-hydroxy amino acid, aldoximes, *aci*-nitro or nitrile oxides, S-alkyl thiohydroximates, thiohydroxamic acids, and desulfoglucosinolates) (Figure 1.18). The first common step in glucosinolate biosynthesis is the conversion of amino acids (**148**) to their respective aldoximes (**149**) (Bennet et al., 1993; 1997; Dawson et al. 1993; Dewick, 1984; Du et al., 1995; Wittstock and Halkier, 2000). The aldoxime-forming enzyme was proposed to catalyze *N*-hydroxylation of amino acids to the corresponding *N*-hydroxyamino acids that decarboxylate to produce the aldoximes (**149**). The cytochrome P450 family types of enzymes were shown to catalyze such transformations (Halkier and Møller, 1991; Koch et al., 1992). The enzymes CYP79F1 and CYP79F2 metabolize chain-elongated derivatives of methionine to their respective aldoximes in the biosynthesis of aliphatic glucosinolates, but not tyrosine, tryptophan, or their derivatives (Chen et al., 2003; Hansen et al., 2001a; Reintanz et al., 2001). CYP79A2 on the other hand catalyzes the metabolism of phenylalanine to phenylacetaldoxime in the biosynthetic pathway of aromatic glucosinolates (Wittstock and Halkier, 2000). The enzymes CYP79B1, CYP79B2 and CYP79B3 catalyze the conversion of *S*-tryptophan (**42**) to indolyl-3-acetaldoxime (**43**) in the biosynthesis of

indolyl glucosinolates (Hansen and Halkier, 2005; Hull et al., 2000; Mikkelsen et al., 2000; Naur et al., 2003b).

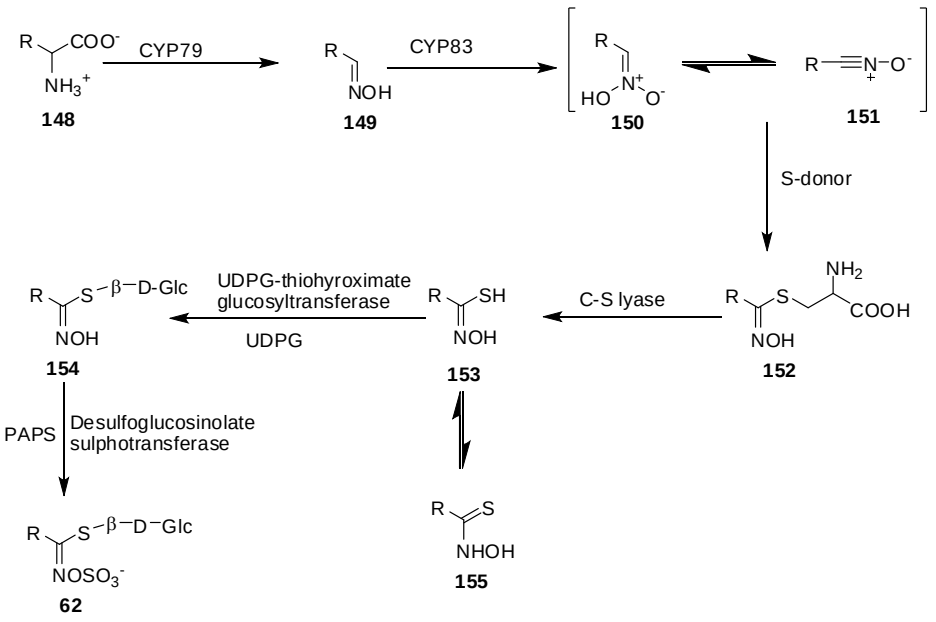


Figure 1.18 Biosynthetic pathway of the functional group glucosinolate (Halkier and Gershenzon, 2006).

Over expression of CYP79B2 in *A. thaliana* resulted in significant increase in levels of indolyl glucosinolates, glucobrassicin (**86**) and 4-methoxyglucobrassicin (**90**), but not of aliphatic or aromatic glucosinolates (Mikkelsen et al., 2000). The aldoxime metabolizing enzymes in the biosynthesis of glucosinolates (in *A. thaliana*) have been characterized. The enzyme CYP83A1 metabolizes aliphatic aldoximes much more efficiently than CYP83B1 (Bak and Feyereisen, 2001; Hemm et al., 2003; Naur et al., 2003a). The enzyme CYP83B1 catalyzes the metabolism of aromatic aldoximes derived from tyrosine and phenylalanine (Hansen et al., 2001b), as well as indolyl-3-acetaldoxime (Bak and Feyereisen, 2001). CYP83A1 enzyme is capable of

metabolizing indolyl-3-cetaldoxime albeit with low efficiency (Bak and Feyereisen, 2001). Conjugation of the aldoxime (**149**) with the thiol donor requires conversion of **149** into a reactive intermediate that can be attacked by the nucleophilic thiol donor. The CYP83 enzymes have been proposed to catalyze the oxidation of respective aldoximes to highly reactive 1-*aci*-nitro compounds (**150**) or nitrile oxides (**151**) (Bak et al., 2001; Bak and Feyereisen, 2001; Hansen et al., 2001b). Following its formation, the intermediate is conjugated with a suitable thiol donor to yield an *S*-alkyl-thiohydroximate (**152**), which can happen non-enzymetically. For decades, both cysteine and methionine have been thought to be thiol donors in the biosynthetic pathway of many secondary metabolites; the former is a more efficient donor than the later (Mikkelsen et al., 2002). However, a recent result has shown that glutathione (GSH) is more likely the sulfur donor (Bednarek et al., 2009 and Schlaeppi et al., 2008). Proper conjugation of the sulfur donor with the reactive intermediate (*aci*-nitro compound or nitrile oxide) is thought to be catalyzed by a glutathione-*S*-transferase type enzyme (Mikkelsen et al., 2004). The *C*-*S* bond of *S*-(thiohydroximoyl)-*L*-cysteine (**152**) is hydrolyzed by *C*-*S* lyase to form thiohydroxamic acid (**153**) which is the next intermediate in the biosynthetic pathway of glucosinolates. The thiohydroxamic acid (**153**) formed is subsequently glycosylated by a nucleotide to produce desulfoglucosinolate (**154**). Pedras and Okinyo have demonstrated the incorporation of labeled indolyl-3-acetohydroxamic acid (**158**) in glucobrassicin (**86**) (Pedras and Okinyo, 2008) (Figure 1.19). Uridine diphosphate glucose (UDPG) was found to be the most efficient glucose donor (Mitsuo and Underhill, 1971) for the conversion of thiohydroxamic acid (**158**) to desulfoglucobrassicin (**159**). A putative UDPG: thiohydroximate glucosyltransferase in the biosynthetic pathway of benzylglucosinolatewas isolated from *A. thaliana* and was characterized as UGT74B1 (Grubb et al., 2004). Loss of *UGT74B1* gene function only caused partial reduction of

glucosinolate accumulation, suggesting that additional enzymes catalyzing glucosylation of thiohydroximates are present (Grubb et al., 2004). UDPG: thiohydroximate glucosyltransferase purified from *B. napus* seedlings were reported to glycosylate sodium 3-phenylpropanothiohydroximate (**156**) to phenylethyl desulfoglucosinolate (**157**) (Reed et al., 1993). The use of similar enzyme preparations with sodium 2-(3-indolyl)acetothiohydroximate (**158**) were reported to give only detectable amounts of desulfoglucobrassicin (**159**) which was suggested not to be stable in the buffer used (pH 6) (Figure 1.19) (Reed et al., 1993).

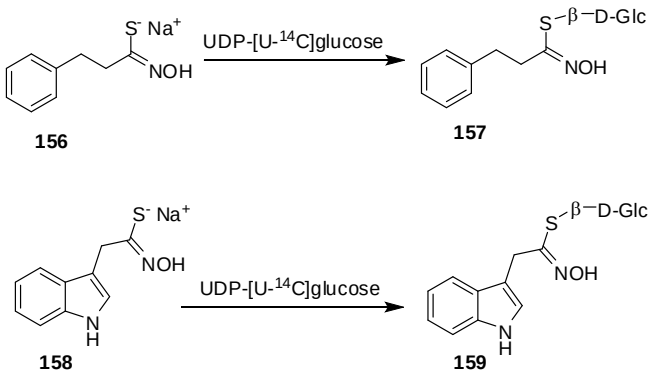


Figure 1.19 UDPG:thiohydroximate glucosyltransferase glucosylation of sodium 3-phenylpropanothiohydoximate (**156**) to phenylethyl desulfoglucosinolate (**157**) and sodium 2-(3-indolyl)acetothiohydroximate (**158**) to desulfoglucobrassicin (**159**) (Reed et al., 1993).

The last common step in building the glucosinolate group (**62**) (for aliphatic, aromatic and indolyl glucosinolates) is the introduction of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to desulfoglucosinolate (**154**). Sulfation of desulfoglucosinolates is catalyzed by sulfotransferases (Glending and Poulton, 1988; 1990; Jain et al., 1989; 1990; Klein et al., 2006; Piotrowski et al., 2004). Three sulfotransferases AtST5a, AtST5b, and AtST5c exhibiting broad substrate range have

been characterized from *A. thaliana* (Piotrowski et al., 2004). AtST5b and AtST5c had higher affinity for aliphatic glucosinolates, whereas AtST5a preferred tryptophan and phenylalanine-derived desulfoglucosinolates (Piotrowski et al., 2004).

(iii) Side chain modification and secondary modifications

Glucosinolate side chain modifications affect the biological activity of their hydrolysis product (Halkier and Gershenzon, 2006). The side chain modifications have been noted to occur in glucosinolates derived from methionine and its homologs (**160**), the transformations may involve sequential oxidations, eliminations, alkylations, and esterifications (Figure 1.20) (Halkier and Gershenzon, 2006; Grubb and Abel, 2006). The sulfur can be successively oxidized to methylsulfinylalkyl (**161**) and methylsulfonylalkyl (**162**) moieties (Halkier and Gershenzon, 2006). Further modifications may involve oxidative cleavage of the methylsulfinylalkyl moiety to yield alkenyl (**163**) or hyroxyalkyl (**164**) side chains for which controlling genetic loci are known in Arabidopsis and Brassica species (Halkier and Gershenzon, 2006). Conversion of glucobrassicin (**86**) to 4-hydroxyglucobrassicin (**88**) followed by methoxylation to form 4-methoxyglucobrassicin (**90**) and glucobrassicin (**86**) to 1-methoxyglucobrassicin (**89**) affect the biological roles in cruciferous defense (Figure 1.21) (Pedras et al., 2010).

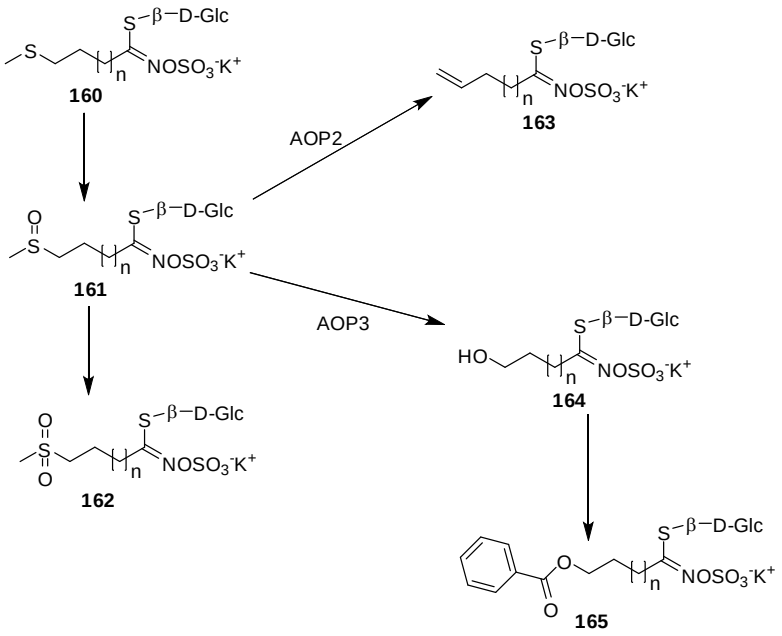


Figure 1.20 Side chain modification of methionine-derived glucosinolates with varying side chain lengths. APO2 and APO3 are putative enzymes catalyzing the reactions in *A. thaliana* (Halkier and Gershenzon, 2006; Kliebenstein et al., 2001).

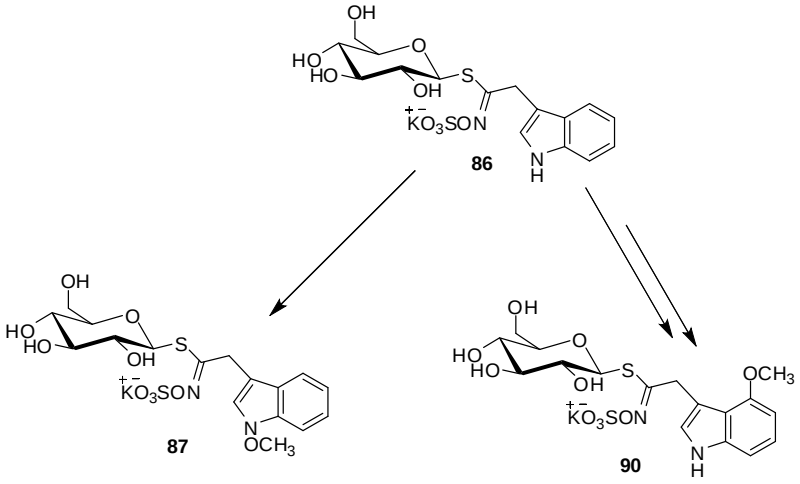


Figure 1.21 Side chain modification: conversion of glucobrassicin (**86**) to 4-methoxyglucobrassicin (**90**) and 1-methoxyglucobrassicin (**87**) (Pedras et al., 2010).

Biosynthetic relationship between cruciferous glucosinolates and phytoalexins

Similar to the biosynthesis of indolyl glucosinolates, the amino acid (*S*)-tryptophan (**42**) also acts as the primary precursor for cruciferous phytoalexins. In fact, it has been reported that indolyl glucosinolates act as the intermediates between (*S*)-tryptophan (**42**) and cruciferous phytoalexins. Incorporation of [2,2´,4,5,6,7-$^{2}H_{6}$]glucobrassicin (**86a**) to hexadutarated 1-methoxybrassinin (**34a**) in salt cress is strong evidence of involvement of indolyl glucosinolates in the biosynthetic pathway of cruciferous phytoalexins (Figure 1.22) (Pedras et al., 2010).

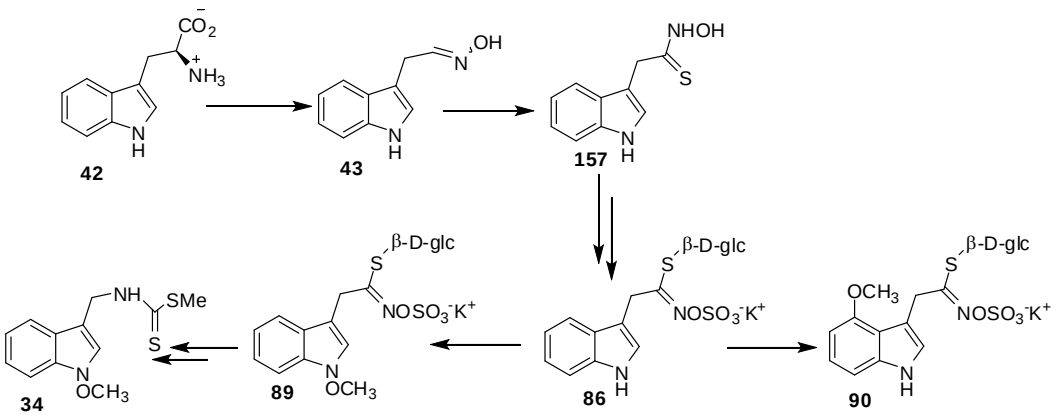


Figure 1.22 Biosynthetic relationship between indolyl glucosinolates and crucifrous phytoalexins (Pedras et al., 2010).

It has been suggested that the formation of cruciferous phytoalexins from indolyl glucosinolates is likely to happen via indolyl isothiocyates (Pedras et al., 2009c; Monde et al., 1994). The incorporation of glucobrassicin (**86**) to 1-methoxybrassinin (**34**) can be explained by the conversion of glucobrassicin (**86**) to 1-methoxyglucobrassicin (**89**), which is then incorporated into 1-methoxybrassinin (**34**)

via 1-methoxyindolyl-3-isothiocyanate (**97**). Incorporation of three compounds 1-acetylindolyl-3-methylisothiocyanate (**166**), 1-*t*-butoxycarbonylindolyl-3-methylisothiocyanate (**167**), and benzyl isothiocyanate (**170**) to 1-acetyl brassinin (**168**), 1-*t*-butoxycarbonylbrassinin (**169**) and methylbenzyl dithiocarbamate (**171**) respectively also supports this hypothesis very strongly (Pedras et al., 2009c; Monde et al., 1994).

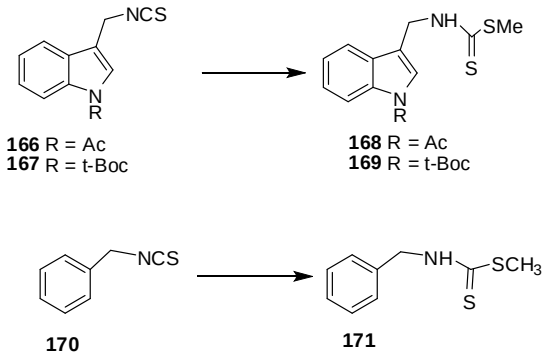


Figure 1.23 Demonstration of biosynthetic relationship between glucosinolates and crucifrous phytoalexins through isothiocyantes (Pedras et al., 2009c and Monde et al., 1994).

Biological activities and metabolism of cruciferous phytoanticipins

Phytoanticipins of cruciferous plants (glucosinolates and derivatives) have been reported to display different types of biological activities; however, several cruciferous specialist insects can overcome glucosinolates by metabolizing these compounds.

Biological activities

Glucosinolates and their breakdown products are known for their important biological activities such as goitrogenic, antinutritional and cancer preventing activities

(Fahey et al., 2001; Keck and Finely, 2004; Fenwick and Heaney, 1983; Fahey et al., 2001; Traka and Mithen, 2009; Vig et al., 2009; Hopkins et al., 2009). It was also found that high level of glucosinolates were associated with resistance of cabbage to fungal pathogen *Peronospora parasitica* (Pers. ex Fr.) (Osbourn, 1996, Greenhalgh and Mitchell, 1976). Transgenic *A. thaliana* (expressing CYP79D2) accumulating aliphatic isopropyl and methylpropyl glucosinolates showed enhanced resistance against the bacterial pathogen *Erwinia carotovora* ssp. *Carotovora* (Ecc). *A. thaliana* (expressing CYP79A1 and CYP79A2) accumulating 4-hydorxybenzyl (**72**) and benzylglucosinolate (**66**) respectively, showed increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. tomato (Brader et al., 2006). On the other hand, CYP79A1 and CYP79A2 mutants showed enhanced susceptibility towards the fungal pathogen *A. brassicicola* and CYP79D2 mutants did not show any differences relative to the wild type. The increasing susceptibly toward *A. brassicicola* in mutants CYP79A1 and CYP79A2 is due to the suppression of jasmonate-dependent defense system that is important for *A. brassicicola* resistance (Brader et al., 2006). A general correlation trend was observed between the extent of pathogen induced production of indole glucosinolates on *B. napus* and resistance to *S. sclerotiorum* (Li et al., 1999).

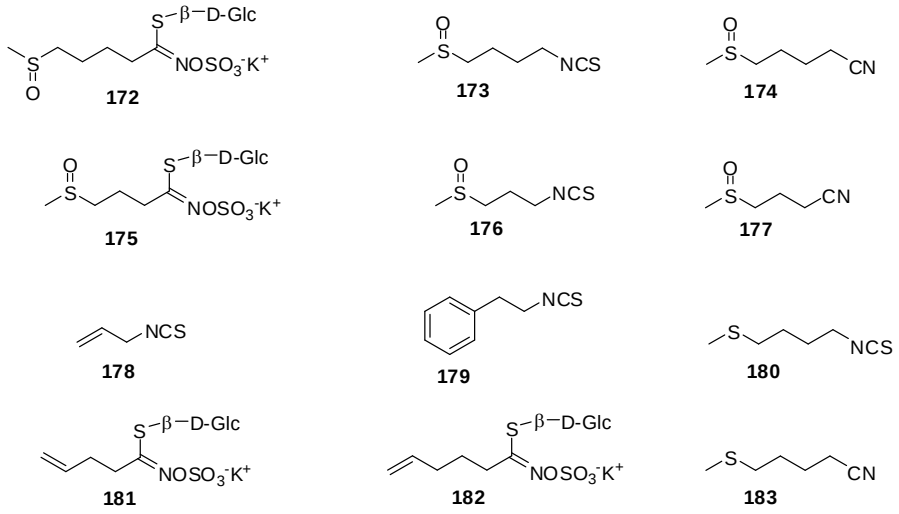
Glucosinolate breakdown products, especially isothiocyanates, have been reported for high antifungal activity against different plant fungal pathogens (Manici et al., 1997; Koenraad et al, 2001; Góralska et al., 2009). From a comparative study of the antifungal activity of several glucosinolate breakdown products, it was shown that aromatic isothiocyanates are more potent than aliphatic isothiocyanates against various fungal pathogens. A general trend was noted in which the fungal toxicity of aliphatic isothiocyanates decreased with increasing side chain length (Manici et al., 1997; Mithen et al., 1986; Sarwar et al., 1998). Although these studies showed that the

various breakdown products of glucosinolates had antimicrobial activity, additional evidence for their significant role in protecting plants against various pathogens was shown in the model plant *A. thaliana* (Tierens et al., 2001). In their investigation, Broekaert and co-workers found that 4-methylsulphinylbutyl isothiocyanate (**173**), a degradation product of 4-methylsulphinylbutyl glucosinolate (**172**), accumulated in *A. thaliana* wild type leaves but was absent in glucosinolate-deficient *gsm1-1* mutant. 4-Methylsulphinylbutyl isothiocyanate (**173**) displayed antimicrobial activity against a number of fungi and bacteria in growth inhibition bioassays (Tierens et al., 2001). Manici and co-workers established that 3-methylsulfinylpropyl isothiocyanate (**176**) could completely inhibit the growth of the plant pathogens *Fusarium culmorum* (W.G. Smith) Sacc., *R. solani, S. sclerotiorum, Diaporthe phaseolorum* var. *caulivora,* and *Pythium irregulare* Buisman, whereas the parent compound glucoiberin (3-methylsulfinylpropyl glucosinolate, **175**) was inactive towards these fungi (Manici et al., 1997; 2000). Volatiles from macerated leaves of genotypes of the crucifers, black mustard (*B. nigra*) and Indian mustard (*B. juncea*), were shown to inhibit fungal growth and sporulation in two plant pathogens, *Helminthosporium solani* Dur. and Mont. and *Verticillium dahliae* Kleb. (Mayton et al., 1996; Olivier et al., 1999); whereas cultivars of black and Indian mustard predominantly produce allyl glucosinolate, sinigrin (**131**) (Daxenbichler et al., 1991, Olivier et al., 1999). GC analyses of their macerated leaf volatiles showed that allyl isothiocyanate (**178**) (enzymatic hydrolysis product of **131**) was the major volatile component and that its production significantly varied in different genotypes (Olivier et al., 1999). The fungicidal activity of the genotypes investigated was reported to correlate with the production of allyl isothiocyanate (**178**); for example, genotypes producing higher amounts of allyl isothiocyanate also exhibited higher fungitoxicity (Olivier et al., 1999). It was shown that allyl isothiocyanate (**178**) could reduce the incidence of blue mould caused by *Penicillium expansum* Link by as

much as 90% (Mari et al, 2002), thus making it a potential biofungicide that could be used to control the devastating effects of this pathogen. Allyl isothiocyanate (**178**) also showed remarkable fungitoxity towards *L. maculans*, an economically important pathogen of crucifers (Mithen et al., 1986). Mithen and co-workers (1986) investigated the antifungal activity of glucobrassicin (**86**) and its degradation products against *L. maculans*. From these investigations, it was reported that a mixture of glucobrassicin and myrosinase significantly inhibited fungal growth in the cultures compared to glucobrassicin (**86**) alone (Mithen et al., 1986). In antimicrobial bioassays using phenylethyl isothiocyanate (**179**), a degradation product of phenylethylglucosinolate (**67**), **179** inhibited the growth of a range of fungi, oomycetes and bacteria (Smith and Kirkegaard, 2002). The results obtained supported the possible application of these compounds in biofumigation to control soil pathogens.

Indolyl glucosinolates and their derivatives were reported to play important roles in plant defense against pathogens (Agerbirk et al., 2009; Clay et al., 2009; Bednarek et al., 2009). Breakdown of 4-methoxyglucobrassicin (**90**), was found to be required to induce defense of *A. thaliana* against the grass powdery mildew *Blumeria graminis* hordei. Bednarek et al. reported that *A. thaliana CYP81F2* gene, encodes a P450 monooxygenase essential for the pathogen-induced accumulation of 4-methoxyglucobrassicin (**90**). 4-Methoxyglucobrassicin (**90**) was found to be activated by the atypical PEN2 myrosinase for antifungal defense (Bednarek et al., 2009). Ausubel and co-workers found that glucosinolates and metabolites, especially 4-methoxyglucobrassicin (**90**) was required for *A. thaliana* innate immune response. 4-Methoxyglucobrassicin (**90**) was reported as responsible for induction of callose, a β(1,3)-glucan polymer strengthens and restrain weak or compromised sections of plant cell wall at the site of pathogen attack. 4-Methoxyglucobrassicin (**90**) induced callose

deposition in *A. thaliana* seedlings as a response to the treatment with Flg22, a synthetic derivative of the microbe-associated molecular pattern (MAMP) polypeptide flagellin (Clay et al. 2009). In both cases, 4-methoxyglucobrassicin (**90**) was reported as playing important role against fungal pathogens but the direct interaction of 4-methoxyglucobrassicin (**90**) with fungal pathogen has not been established.



Glucosinolates have different effects on different insect species. For example, they are deterrent to some insect species and, on the other hand, feeding stimulants to some insect species. These activities include stimulation of insect feeding and oviposition, direct plant defense by acting as feeding deterrents (including mammals, birds), and indirect defense by providing cues to natural enemies of the herbivores (Baker et al., 2006; Halkier and Gershenzon, 2006; Rask et al., 2000). Increasing the glucosinolate levels in *A. thaliana* reduced feeding by a generalist herbivore *Trichoplusia ni* (Hübner), while the specialist *Plutella xylostella* L. (Lepidoptera, Plutellidae) was unaffected by glucosinolate concentration (Kliebenstein et al., 2002). On the other hand, increased glucosinolate levels in *B. rapa* decreased feeding by both

specialist, *Pieris rapae* (Linnaeus). and generalist, *T. ni* (Stowe, 1998). Glucosinolates and their volatile degradation products that protect plants against some herbivores may also attract specialized herbivores or serve as oviposition stimulants (Gabrys and Tjallingii, 2002; Mewis et al., 2002). Sinigrin (**131**), for example acted as a feeding deterrents to *Acyrthosiphon pisum* Scop. (pea aphid), but attracted *Brevicoryne brassicae* L. (cabbage aphids) to a non-host plant (Gabrys and Tjallingii, 2002). Non-host plants treated with extracts from host plants stimulated oviposition behavior of cabbage webworm, *Hellula undalis* (Fabricius) (Lepidoptera: Pyralidae) (Mewis et al., 2002). Chemical analysis of the host plant (white mustard) extracts revealed that it predominantly contained aromatic glucosinolates, sinalbin (4-hydroxybenzylglucosinolate) (**72**) and benzylglucosinolate (**66**), unlike the less preferred plants that contained higher amounts of aliphatic and indolyl glucosinolates (Mewis et al., 2002). Stimulation of oviposition in cabbage root fly (*Delia radicum*) was attributed to indolyl glucosinolate, glucobrassicin (**86**) (Roessingh et al., 1992), whereas both aliphatic alkenyl glucosinolates 3-butenyl- (**181**) and 4-pentenylglucosinolates (**182**) and glucobrassicin (**86**) stimulated oviposition behavior in turnip root fly (*Delia floralis* (Fallen)) (Simmonds et al., 1994). Both flies possessed chemoreceptors that responded to different glucosinolates, the response was correlated to the number of eggs laid (Roessingh et al., 1992; Simmonds et al., 1994). Sinigrin (**131**), sinalbin (**72**), and allyl isothiocyanate (**178**) triggered oviposition-probing behavior of turnip sawfly (*Athalia rosae* L.), these findings indicated that both glucosinolates and their degradation products play a significant role in host-finding and oviposition among insects (Baker et al., 2006). The volatile isothiocyanates derived from short chain aliphatic glucosinolates may serve to attract insects from a distance and help them to locate the host plant, whereas glucosinolates within leaf surface waxes (e.g. indolyl glucosinolates) may serve as stimulants for egg laying (Giamoustaris and

Mithen, 1995; Renwick, 2002). Various studies have demonstrated that plants respond to insect herbivory by accumulating higher levels of glucosinolates presumably to increase the plants resistance to subsequent attacks (Agrawal et al., 2002; Mewis et al., 2005; Traw, 2002; van Dam et al., 2005). The levels of sinigrin (**131**) and glucobrassicin (**86**) were found to increase by 19 and 16%, respectively, in black mustard when fed on by white butterfly (Traw, 2002). Some plants use glucosinolate degradation products to protect themselves against specialist herbivores. For example, allyl isothiocyanate (**178**), reduced the growth and survival of the crucifer specialist, *Pieris rapae* L. (white butterfly) (Agrawal and Kurashige, 2003). Although glucosinolates and their degradation products may be advantageous to specialist herbivores (e.g. host-recognition and feeding stimulation), they are deleterious to generalists. Sinigrin (**131**) and its allyl isothiocyanate (**178**) degradation product were found to be lethally toxic to the crucifer generalist *Spodoptera eridania* (Cramer) (southern armyworm), whereas the specialist *P. xylostella* (cabbage moth) was only affected by allyl isothiocyanate (**178**) (Li et al., 2000). Developing plant lines with increased levels of glucosinolates resulted in greater damage by the specialists, while reducing grazing levels by the generalists (Giamoustaris and Mithen, 1995). *B. juncea* lines with high glucosinolate concentration were much better protected from the larvae of generalist herbivore *S. eridania* than lines with low glucosinolate concentration (Li et al., 2000). On the other hand, the Brassicaceae specialists seemed to respond to particular glucosinolates, suggesting that the plant glucosinolate profile is more important for plant-insect interaction rather than the total glucosinolate concentration (Giamoustaris and Mithen, 1995).

The defense resulting from the glucosinolate-myrosinase system has mainly been credited to the isothiocyanates produced from glucosinolates. The toxicity of

isothiocyates against several insects has been found to be comparable to that of many commercial insecticides (Winde and Wittstock, 2011). It was found that simple nitriles are less toxic to insects than the corresponding isothiocyanates (Wittstock et al., 2003). Larvae of generalist lepidopteran herbivore *Spodoptera littoralis* (Boisduval) develop faster on nitriles (**174**, **177**, and **183**) than on isothiocyanates (**173**, **176**, and **180**) producing transgenic *A. thaliana* lines (Burow et al., 2006).

Metabolism

Counteradaptation of glucosinolate defense by specialist insects of crucifers is well known. Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system have been reviewed recently by Winde and Wittstock (Winde and Wittstock, 2011). Cruciferous specialist insects have been reported to use different strategies to overcome glucosinolate-derived defense of plants. Several strategies have been found in specialist insects to adapt to plant defense compounds including (1) avoidance of cell disruption, (2) rapid absorption of intact glucosinolates, (3) rapid metabolism of glucosinolates to harmless compounds that are not substrate for myrosinases, and (4) diversion of plant myrosinase-catalyzed glucosinolate hydrolysis (Winde and Wittstock, 2011). The insects that sequester glucosinolates use them for their own protection by deterring predators such as birds, lizards and ants (Halkier and Gershenzon, 2006). Diversion of aglycons produced from glucosinolates by myrosinase to less toxic compounds was found to be most common in several different specialist insects of crucifers.

The specialist insect herbivores of glucosinolate containing plants, *P. rapae* can overcome the plant glucosinolate-myrosinase defense mechanism by converting the aglycon **89**, produced by myrosinase: instead of producing the toxic isothiocyanates,

the hydrolysis is directed toward the formation of nitriles. Several aliphatic and aromatic glucosinolates have been reported to be metabolized by larvae of the specialist *P. rapae* (cabbage white butterfly) to the corresponding nitriles (Figure 1.24), which was then excreted in their frass (Wittstock et al., 2004). The conversions of glucosinolates to the nitriles are due to presence of a gut protein, nitrile specifier protein (NSP) in the larvae. Presence of nitrile specifier protein (NSP) was also reported in several other *Pieris* species specialized on glucosinolate containing plants (Wheat et al., 2007).

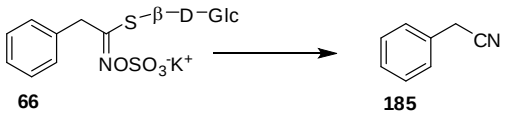


Figure 1.24 Metabolism of glucosinolates by *Pieris rapae* (Wittstock et al, 2004 and Agerbirk et al., 2006).

Agerbirk and co-workers found that 4-hydroxybenzylglucosinolate (**72**) also known as sinalbin was metabolized by several cruciferous insects first to 4-hydroxyphenyl acetonitrile (**184**) and finally sulfated to the corresponding sulfo acetonitrile (**187**) (Figure 1.25) (Agerbirk et al., 2006). Similar results were also reported by Müller and co-workers (Müller et al., 2003).

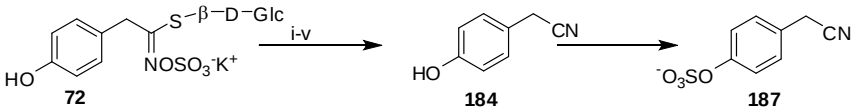


Figure 1.25 Metabolism of 4-hydroxybenzylglucosinolate(**72**, sinalbin) by (i) *Pieris rapae*, (ii) *Anthocharis cardamines*, (iii) *Pieris virginiensis*, (iv) *Pieris napi oleracea*, and (v) *Pieris brassicae* (Agerbirk et al., 2006).

Hydroxyphenylalkyl glucosinolates, as well as the corresponding O-methyl derivatives, are metabolized to sulfates of the corresponding nitriles, suggesting that larval O-demethylase and sulfotransferase activities are also involved beside the nitrilase in the metabolic pathway in *P. rapae* (Agerbirk et. al., 2010). Additional metabolism through β-hyrdroxylation of the aliphatic part of the nitrile was also observed (Figure 1.26).

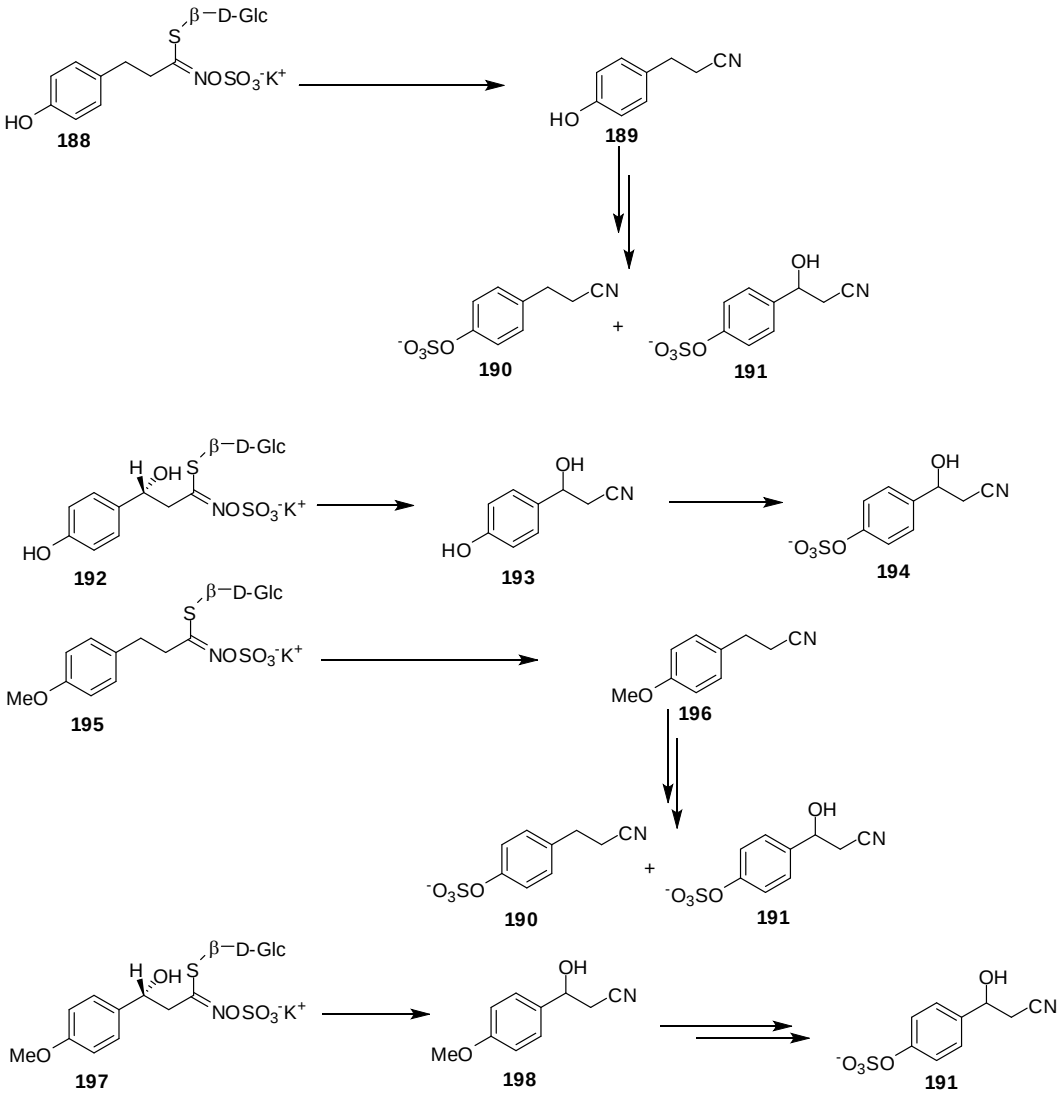


Figure 1.26 Metabolism of aromatic glucosinolates by *Pieris rapae* (Aberbirk et al., 2010).

Wittstock and co-workers have demonstrated that phenyl acetonitrile (**185**) formed from benzylglucosinolate (**66**) was further metabolized to phenylacetyl glycine (**200**) by *P. rapae*. Phenyl acetonitrile (**185**) was first transformed to phenyl acetic acid (**199**), which was further metabolized to phenylacetyl glycine (**200**) (Figure 1.27). (Vergara et al., 2006).

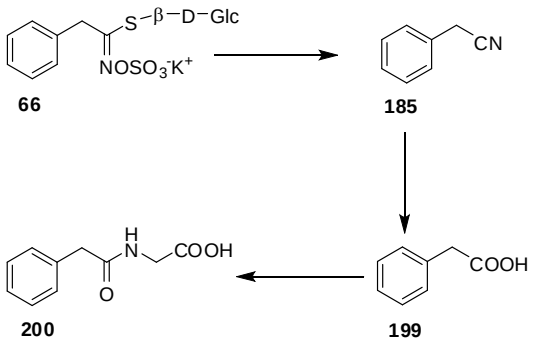


Figure 1.27 Metabolism of benzylglucosinolate (**66**) by *Pieris rapae* (Vergara et al., 2006).

On the other hand, a specialist cruciferous feeder *P. xylostella* removes the sulfate from the glucosinolate using a glucosinolate sulfatase to make desulfoglucosinolate, which is no longer a substrate of myrosinase. The larvae of *P. xylostella* metabolize sinigrin (**131**) to its desulfoglucosinolate (**201**) (Figure 1.28) (Ratzka et al., 2002).

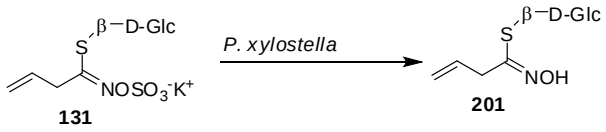


Figure 1.28 Metabolism of sinigrin (**131**) by *Plutella xylostella* (Ratzka et al., 2002).

In contrast to the specialist insect *P. xylostella*, which produces constitutive glucosinolate sulfatase, desert locusts *Schistocerca gregaria*, a generalist, possesses an inducible glucosinolate sulfatase in the gut that hydrolyzes glucosinolates to their corresponding desulfonated forms. Sulfatase was found throughout the desert locust gut

and was able to hydrolyze all the glucosinolates present in *Schistocerca purpurea* (Forskål). Glucosinolate sulfatase activity is induced tenfold when locusts are fed on *S. purpurea* and activity was reduced when glucosinolates were removed from the locust diet (Falk and Gershenzon, 2007).

Several species of the sawfly genus *Athalia* sequester glucosinolates in their homolymph and thereby circumvent the glucosinolate–myrosinase system. Muller and co-workers have fed labeled glucosinolates and found that the larvae of sawfly *A. rosae* uses sulfatase to metabolize benzylglucosinolate (**66**), phenylethylglucosinolate (**67**), and sinigrin (**131**) to the corresponding desulfoglucosinolate and finally transformed the desulfoglucosinolates to desulfoglucosinolate-3-sulfates (Figure 1.29) (Opitz et al., 2011). Uptake of glucosinolates and their metabolism by *A. rosae* was also reported by Müller and Wittstock (2005). They found that several aromatic and aliphatic glucosinolates were metabolized by *A. rosae*, but no indolyl glucosinolates were metabolized. Only a little amount of desulfoglucosinolates in the larval extract but no other metabolites were detected. They did not observe any indication of presence of myrosinase or sulfatase activity in the protein extract of larval tissues.

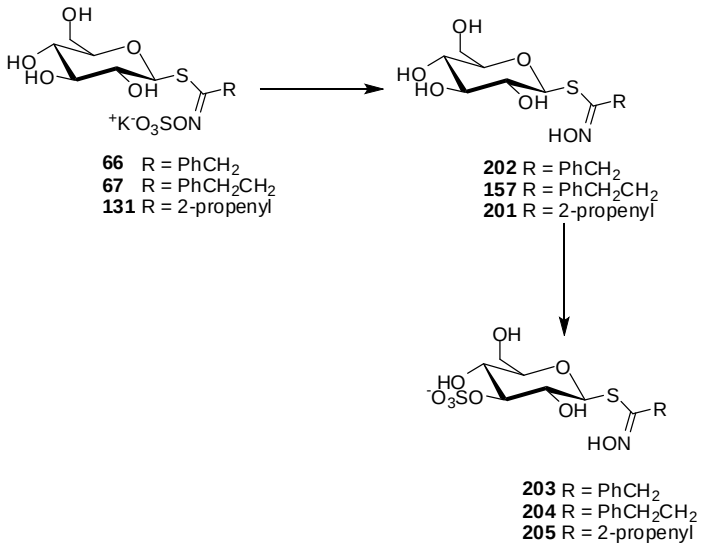


Figure 1.29 Metabolism of glucosinolates (**66**, **67**, and **131**) by *Athalia rosae* (Opitz et al., 2011).

The cabbage aphid, *Brevicoryne brassicae* L., often known as "walking mustard oil bomb" has developed a defense system that exploits and mimics that of its host plants, involving sequestration of glucosinolates and metabolism using its own myrosinase. Like its host plants, the aphid produces a myrosinase to catalyze the hydrolysis of glucosinolates to produce isothiocyanates. Uptake and metabolism of a glucosinolates were investigated when the aphid was fed on glucosinolate producing plants or an in vitro system (Kazana et al., 2007; Francis et al., 2002). Two glucosinolates, progoitrin (**206**) and sinalbin (**72**) was metabolized by *B. brassicae* to the corresponding isothiocyanates, similar results were also found using both crude aphid homogenates and purified myrosinase (Figure 1.30) (Francis et al., 2002).

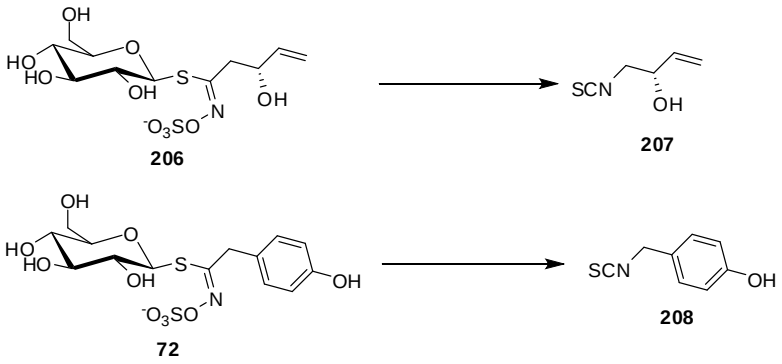


Figure 1.30 Metabolism of glucosinolates (**206** and **72**) by *Brevicoryne brassicae* (Kazana et al., 2007; Francis et al., 2002).

In contrast to specialist insects, generalists use general and broad-range detoxification mechanisms to detoxify toxic metabolites produced in plants (Bernays and Chapman, 2000). A common mechanistic pathway in insects is the formation of glutathione adduct with the toxins using glutathione S-transferases (GSTs). GSTs are a large family of multifunctional enzymes that catalyze the conjugation of electrophilic compounds with the thiol group of reduced glutathione. After conjugation, potentially toxic substances become more water soluble and generally less toxic (Habig et al., 1974). Conjugation with glutathione is a major route by which many isothiocyanates are eliminated in mammals. The glutathione adduct formed with isothiocyanates may be transported out of the cells where they may enter the mercapturic acid pathway for renal excretion or dissociate to release the free isothiocyanate (Traka and Mithen, 2009). Similar types of transformation have been proposed in plant pathogens and insects. Induction of GST activity in response to secondary metabolites from *Brassica* plants has been reported in several generalist insect herbivores: *Spodoptera frugiperda* (Smith), *T. ni* (Wadleigh and Yu, 1988), and *Myzus persicae* (Sulzer) (Francis et al., 2005). The generalist whitefly species *Bemisia tabaci* (Gennadius) is a major

agricultural pest of field and horticultural crops worldwide, including members of the Brassicaceae. Morin and co-workers reported the expression of three GST genes from *B. tabaci* in response to secondary metabolites from a *Brassica* species. One gene, *BtGST2*, was up-regulated when the insect switches to a Brassicaceae host and down-regulated when the insect switches back to a non-Brassicaceae host. This expression pattern of *BtGST2* was related to *B. tabaci* feeding behavior and adaptation to secondary plant toxic substances such as glucosinolate-myrosinase derived isothiocyanates (Alon et al., 2010). Glutathione transferase activity towards various plant isothiocyanates was studied in larvae of the two generalists, *S. frugiperda*, and *T. ni*, and the specialist, *Anticarsia gemmatalis* (Hübner) using their mid gut soluble fractions as the enzyme source. The generalists were found to be adapted to feeding on isothiocyanate-containing crucifers. Allyl and benzyl isothiocyanate were found to be metabolized by glutathione transferase from the two generalist species, but no activity was detected with the specialist. (Wadleigh and Yu, 1988).

Metabolism of glucosinolates by fungi

The induction of *A. brassicicola* glucosyl transferase (AbGst1) by isothiocyanates and the early transient expression of AbGst1 in planta suggested that this protein might be involved in isothiocyanate detoxification in *A. brassicicola* during plant infection (Sellam et al., 2006). Conclusive evidence for conjugation of isothiocyanate with glutathione in insect or fungus is still missing. Excretion of glutathione conjugates or derived metabolites of isothiocyanates have not been found yet in insects or fungal pathogens. A soil isolate of *Aspergillus flavus* Link ex Fr. converted 2-phenylethyl (**67**) and 2-propenylglucosinolate (**131**) and their desulfo-

derivatives **156** and **197**, to nitriles **205** and **206** respectively (Figure 1.31) (Galletti et al., 2008).

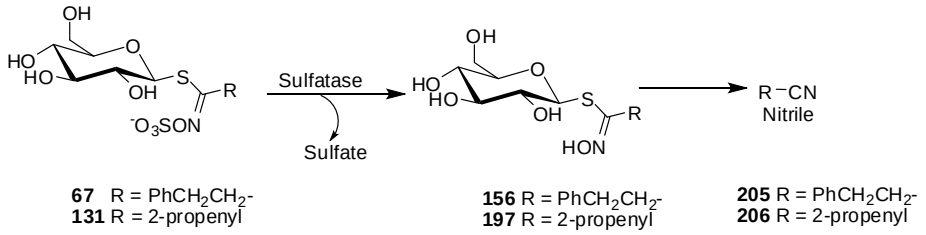


Figure 1.31 Metabolism of glucosinolates (**67** and **131**) by *Aspergillus flavus* (Galletti et al., 2008).

Two other fungal species, *Fusarium oxysporum* (Schlechtendahl f. sp. albedinis), and *Aspergillus clavatus* (Desmazières (Eurotiales: Trichocomaceae)), were also reported to have the ability to metabolize sinigrin (Smits et al., 1993). *A. clavatus* metabolized sinigrin (**131**) to the corresponding nitrile (**206**) and isothiocyanate (**178**) (Figure 1.32) whereas, in case of *F. oxysporum*, the concentration of sinigrin (**131**) decreased slowly but no metabolites were detected. The ability of *A. clavatus* to metabolize glucosinolate was an indication of the presence of myrosinase type enzyme in this species. Formation of isothiocyanate and nitrile is similar to glucosinolate-myrosinase transformation in the plant (Smits et al., 1993).

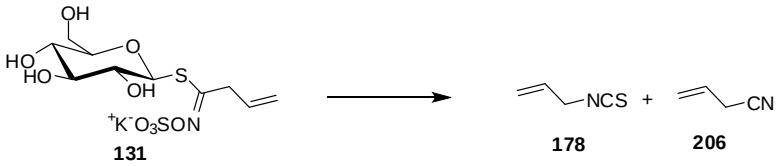
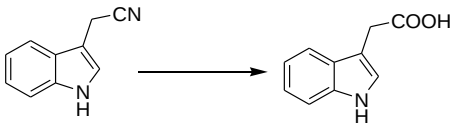


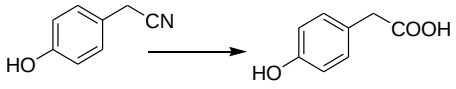
Figure 1.32 Metabolism of sinigrin (**131**) by *Aspergillus clavatus* (Smits et al., 1993).

The ability of fungal pathogens of crucifers to metabolize glucosinolates has not been reported until recently (Pedras and Hossain, 2011), and is part of the work described in this thesis. However, indolyl-3-acetonitrile (**40**) and 4-hydroxyphenylacetonitrile (**182**), two hydrolytic products from glucosinolates are found to be metabolized by some crucifer pathogenic fungi. *L. maculans* (BJ-125), *L. maculans* (Laird 2), *L. maculans* (Mayfair 2), *S. sclerotiorum* (clone # 33) and *R. solani* (AG2-1) converted indolyl-3-actonitrile (**40**) to indolyl-3-acetic acid (**207**). *L. maculans/P. lingam* and *S. sclerotiorum* (clone # 33) converted 4-hydroxyphenylacetonitrile (**182**) to 4-hydroxyphenylacetic acid (**208**) (Figure 1.33 and 1.34) (Pedras and Montaut, 2003).



40 **207**

Figure 1.33 Metabolism of indolyl-3-acetonitrile (**40**) by fungi: *L. maculans* (BJ-125), *L. maculans* (Laird 2), *L. maculans* (Mayfair 2), *S. sclerotiorum* (clone #33), and *R. solani* (AG2-1) (Pedras and Montaut, 2003).



182 **208**

Figure 1.34 Metabolism of 4-hyroxyphenylacetonitrile (**182**) by fungi: *L. maculans* (BJ-125), and *S. sclerotiorum* (clone #33) (Pedras and Montaut, 2003).

1.5. Myrosinases in plants and pathogens

The hydrolysis of glucosinolates is catalyzed by thioglucosidases, also known as myrosinases (EC 3.2.3.1). Myrosinases are *S*-glucosidases whose amino acid sequences display strong similarities with several *O*-glucosidases (Bones and Rossiter, 1996). Based on amino acid sequence similarities, glucosyl hydrolases have been classified into over 70 families. Myrosinases belong to the family of *O*-glucosidases together with prokaryotic and eukaryotic *O*-glucosidases, 6-phosphoglucosidases, 6-phosphogalactosidases, and galactosidases (Rask et al., 2000). Though *O*-glucosidases are extremely widespread in nature, myrosinases are the only known *S*-glucosidases. Myrosinases have been found to be retaining enzymes consistent with the sequence similarity with family 1 *O*-glucosidases. Myrosinases activity was first reported by Bussy in 1840, who studied glucosinolates in *B. nigra* and found a protein, which was involved in the hydrolysis of the glucosinolate sinigrin (**131**). Myrosinases occur in all Brassicaceae species examined so far along with several other plant families (Rodman, 1991). Enzymes with myrosinase activity have also been found in insects, fungi, bacteria commonly associated with human and animal gut microflora (Campbell et al., 1987; Rabot et al., 1993).

1.5.1. Plant myrosinases

In glucosinolate containing plants, myrosinases have been reported to occur in seeds, leaves, stems, and roots. Myrosinases have been found in a special type of cell in the plant known as myrosin cells (Bones and Rossiter, 1996; Bridges et al., 2002; Morant et al., 2008; Winde and Wittstock, 2011). Distribution of myrosinase was found

to be both organ and species specific. Myrosinases from hypocotyls showed the highest specific activity among the seedling organs (Bones, 1990). Myrosinases are glycosylated proteins with various thiol groups, disulfide, salt bridges, and depending on the source, have multiple forms with different molecular weights, number of subunits and percentage of carbohydrates. Sørensen and co-workers (1986) confirmed the presence of 14 isoenzymes in white mustard and a mixture of isoenzymes in *B. nigra*, *B. napus*, and *Sinapis alba* L. (Buchwaldt et al., 1986). Two myrosinases separated from cotyledons of *B. napus* seedling showed different degree of glycosylation (James and Rossiter, 1991). The carbohydrate content varies from 10 to 23%, reported for several myrosinases from *B. napus*, *S. alba*, *B. juncea* (seed), and *W. japonica*. Molecular mass of purified myrosinases varied from 125 kDa to 480 kDa with 2 to 14 subunits (Bones and Rossiter, 1996). Analysis of cDNA clones encoding myrosinases from *S. alba*, *B. napus* and *A. thaliana* showed that the molecular mass of the protein chain in a myrosinase subunit is ca. 60 kDa (Hara et al., 2001). The structure of a native myrosinase from white mustard seed (*S. alba*) was determined by X-ray crystallography. The protein folds into a $(\beta/\alpha)_8$-barrel structure, very similar to that of the cyanogenic β-glucosidase from white clover. The enzyme forms a dimer stabilized by a Zn^{2+} ion and is heavily glucosylated (Brumeister et al. 1997). A hydrophobic pocket was revealed in the myrosinase structure, which may work as a binding site for the hydrophobic side chain of glucosinolates; the presence of two arginine residues ideally suits the active site for the interaction with the sulphate group of the substrate (Brumeister et al. 1997; Kumar et al., 2011). Myrosinases form complexes with other proteins, such as myrosinase-associated protein, and epithiospecifier protein.

Myrosinase genes have been cloned from oilseed rape (*B. napus*), turnip (*B. campestris*), leaf mustard (*B. juncea*), white mustard (*S. alba*), and thale cress (*A. thaliana*) and are classified into four subtypes based on their amino acid sequence (Xue et al., 1992). The isolation of cDNA and genes encoding myrosinases allowed detailed studies of the existence of myrosinase isoforms and their tissue distribution. Studies of cDNA sequences and genomic hybridization patterns in *S. alba* and *B. napus* have shown that myrosinases in Brassicaceae are encoded by a gene family comprising at least three subfamilies, denoted MA (or Myr1), MB (or Myr2) and MC. Analysis of Southern blot data suggested the presence of ca. 5 MA, 10–15 MB and ca. 5 MC genes in *B. napus*. In *S. alba* only MA and MB myrosinase transcripts have been identified so far, and these two subfamilies comprise fewer genes than in *B. napus* (Xue et al., 1992; Rask et al. 2000). Myrosinases that belong to the MA family occur as free dimers (140 kDa), while members of the MB and MC families are found in high molecular complexes (200–1000 kDa), with myrosinase binding proteins (MBP) and the myrosinase associated proteins (MAP) (Rask et al., 2000).

Two myrosinases isolated from *B. napus* have been tested for myrosinase activity on sinigrin (**131**), progoitrin (**202**), phenylethylglucosinolate (**67**), benzylglucosinolate (**66**), glucobrassicin (**86**) and 1-methoxyglucobrassicin (**87**) (James and Rossiter, 1991). Both isoenzymes have shown similar substrate specificities; highest activity against aliphatic glucosinolates (**131** and **202**), medium activities against two aryl glucosinolates (**66** and **67**) and the least activity against two indole glucosinolates (**86** and **87**) (James and Rossiter, 1991). Bjorkman and Lonnerdal (1973) prepared myrosinase isoenzymes from white mustard seed (*S. alba*) and rape seed (*B. napus*) and tested substrate specificity against several aliphatic and aromatic glucosinolates but did not notice any significant differences. Myrosinase from *Crambe*

abyssinica showed uncommon properties and behavior; homogeneous myrosinase was isolated and investigated to establish the important physicochemical features, including kinetic properties using epimers progoitrin (*R*, **202**) and epi-progoitrin (*S*, **209**) as substrates, with and without ascorbate as an activator. Crambe myrosinase was found as highly specific to epi-progoitrin (**209**), which is probably due to a better stabilization of the enzyme−substrate complex. This stabilization was explained by additional hydrogen bonding that only epi-progoitrin (**209**) form between its hydroxyl group and a suitable residue in the hydrophobic pocket where the "docking" of the glucosinolates side chain takes place (Bernardi et al., 2003). Hydrolysis of epi-progoitrin (**209**) leads to the formation of three bio-active enantiomerically pure compounds: (2*S*)-1-cyano-2-hydroxy-3-butene (**211**), (2*S*)-1-cyano-2-hydroxy-3,4-epithiobutane (**212**), and (5*R*)-5-vinyloxazolidine-2-thione (**214**). The activity of Crambe myrosinase (MYRc) towards epi-progoitrin (**209**) is highly specific compared to any other glucosinolates tested. MYRc displayed only a moderate activity towards the progoitrin (**202**), an epimers of epi-progoitrin (**209**), similar to other glucosinolates were tested (Galletti et al., 2001).

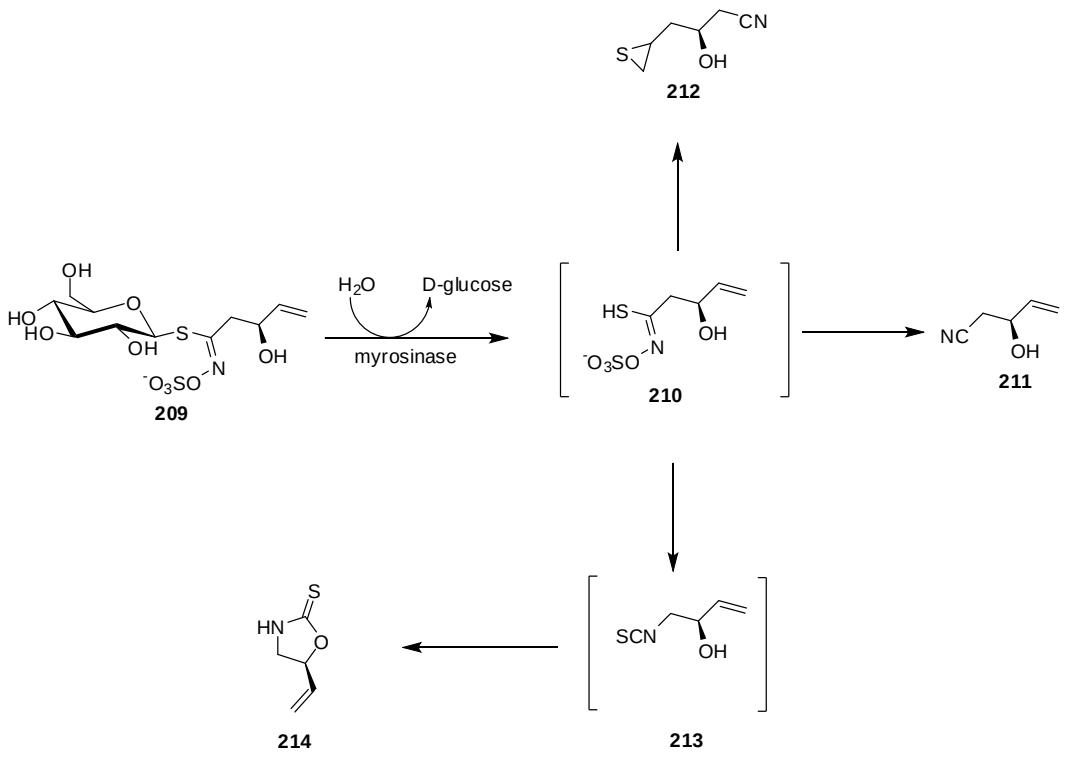


Figure 1.35 Myrosinase catalyzed hydrolysis of epi-progoitrin (**209**) (Bernardi et al., 2003; Galletti et al., 2001).

The myrosinase isolated from *Lepidium sativum* L. was tested against 29 different glucosides but only four were found to be good substrates, two glucosinolates, sinigrin (**131**) and benzylglucosinolate (**66**), but the corresponding α-glucosides were inactive (Botti et al., 1995). Ettlinger et al. have demonstrated that sulfate groups in glucosinolates are essential for myrosinase activity, as desulfoglucosinolates did not act as substrates for myrosinase (Ettlinger et al., 1961). Ascorbic acid acts as a specific activator of myrosinase. The rate of hydrolysis of sinigrin by mustard myrosinase increased more than 25-fold by 1 mM ascorbic acid, but at high concentration ascorbic acid act as inhibitor of the enzyme (Ohtsuru and Hata, 1979).

The stereochemistry of hydrolysis of sinigrin by *S. alba* myrosinase was followed by ^{1}H NMR and the enzyme was found to operate with a mechanism retaining the anomeric configuration at the cleavage point exactly like the related O-glycosidases. Myrosinase functions via a mechanism that requires two steps: the formation of the glucosyl-enzyme with concomitant aglycon departure, and hydrolysis of glucosyl-enzyme by a water molecule (Burmeister et al., 1997; Botti et al., 1995).

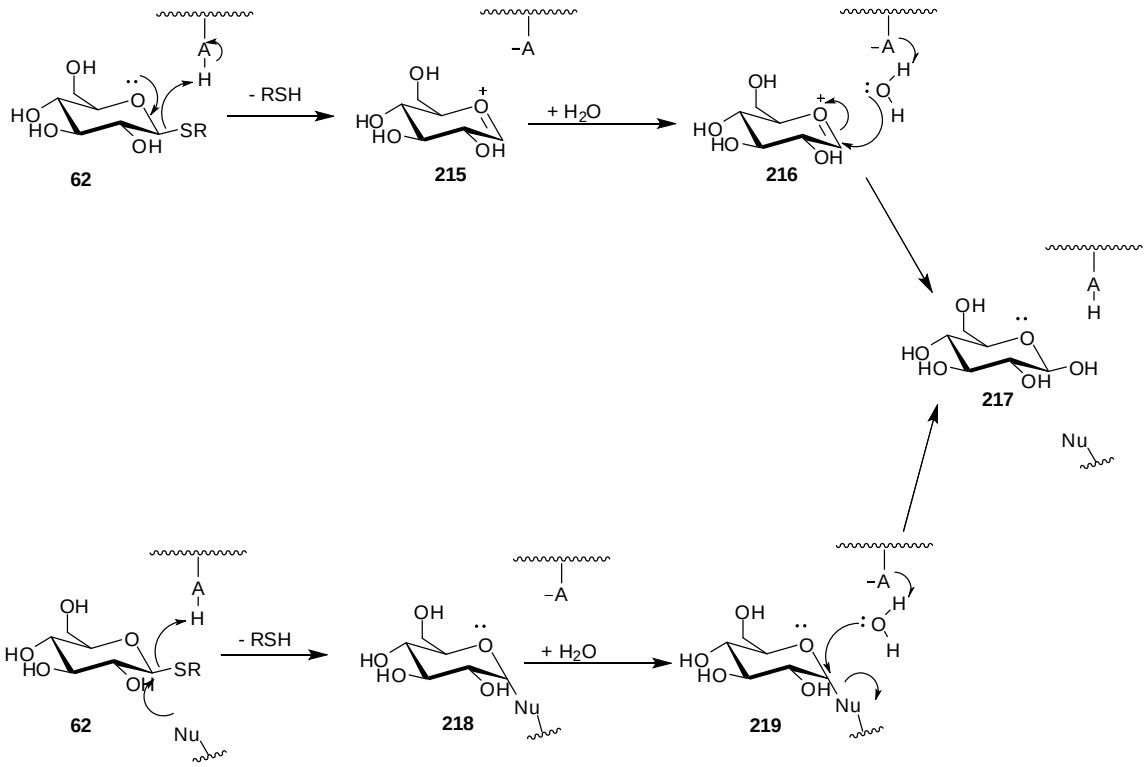
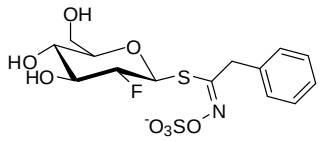


Figure 1.36 Proposed mechanism of hydrolysis catalyzed by myrosinases (Botti et al., 1995).

The presence of the hydroxyl group at C-2 of the glucose is necessary for the enzymatic activity as myrosinase was readily inactivated by 2-deoxy-2-fluoroglucotropaeolin (**220**) (Cottaz et al., 1996).



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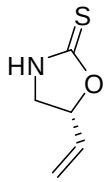
1.5.2. Aphid myrosinases

The first activity for an aphid myrosinase was observed in *Brevicoryne brassicae* L. when goitrin (5-vinyl-2-oxazolidinethione, **221**), the hydrolysis product of progoitron (2-hydroxy-3-butenyl glucosinolate, **202**) was found in crushed *B. brassicae,* which had been feeding on *B. napus* (MacGibbon and Allison 1968). Further work by this group has shown that the myrosinase also occurs in the turnip aphid, *Lipaphis erysimi* (Kaltenbach) and activity in both cases was restricted to the head and thorax regions. *L. erysimi* showed consistently lower levels of myrosinase activity than *B. brassicae* (MacGibbon and Beuzenberg, 1978). Aphid myrosinase is localized to the sarcoplasm of non-flight muscle, but the location of sequestered glucosinolate within aphids has not been determined. Interestingly, Rossiter and co-workers later reported that similar to the host plant, both aphids (*B. brassicae* and *L. erysimi*) posses compartmentalization of glucosinolate and myrosinase. They have found that *B. brassicae* (cabbage aphid) and *L. erysimi* (turnip aphid) can sequester glucosinolates from their host plants avoiding the generation of toxic degradation products by compartmentalizing myrosinase (thioglucosidase) into crystalline microbodies. They have proposed that death or damage to the insect by predators or disease may cause disruption of compartmentalized myrosinase resulting in the formation of toxic metabolites (Bridges et al., 2002).



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The myrosinases from *B. brassicae* and *L. erysimi* are significantly different from plant myrosinases (Jones et al. 2001, 2002; Pontoppidan et al. 2001; Bridges et al. 2002; Husebye et al. 2005). Jones et al. (2001) reported the first non-plant myrosinase from the cabbage aphid *B. brassicae* to homogeneity. The protein is a dimer with subunits of mass 54 kDa. Unlike the plant myrosinase the aphid enzyme does not appear to be a glycoprotein and is not activated by ascorbic acid. The apparent Km of the aphid myrosinase was 0.613 and 0.915 mM respectively for sinigrin (**131**) and benzylglucosinolate (**66**) indicating that the enzyme has a greater affinity for allyl glucosinolate (Jones et al., 2001; Kazana et al., 2001). The enzyme was inhibited by ascorbate at the concentration that normally activates plant myrosinases. The presence of myrosinase in an insect pest specialist may be an example of a co-evolution process that facilitates host specialization (Pontoppidan et al., 2001).

1.5.3. Microbial myrosinases

A large number of microorganisms such as *Escherichia coli, Lactobacillus acidophilus, Bacillus cereus, Enterobacter cloacae* and several *Aspergillus* species have been reported to show myrosinase activity. Staron (1974, 1975) has reported transformation of glucosinolates from rapeseed by yeast *Geotrichum candidum* which was due to the presence of a myrosinase. Several other microorganisms possessing myrosinase activity have been described, such as *E. coli* (Oginsky et al., 1965), *E.*

cloacae (Tani et al., 1974), *B. cereus* (Huber et al., 1983), *Sphingobacterium* sp. strain OTG (Meulenbeld and Hartmans, 2001), and *Aspergillus niger* v. Tieghem (Ohtsuru et al., 1973). Myrosinase activity in *Aspergillus* species have been reported by several groups (Reese et al., 1958; Sakorn et al., 1999, 2002; Ohtsuru et al., 1969, 1973; Smits et al., 1993). However, only in few cases have the myrosinases from these organisms been characterized.

Myrosinase activity could not be detected in the supernatant of the fungal cultures of *A. clavatus*, but was found in cell free extracts. This indicates that this myrosinase is a cytosolic enzyme, and that the degradation of sinigrin is an intracellular process (Smits et al., 1993). Myrosinase activity was determined using the supernatant of ground mycelia of fungi, with a strong activity found in the mycelia of *A. niger* AKU 3302, but only slight activity was detected in *Aspergillus sydowii* (Thom et Church) IFO 4284 (Ohtsuru et al., 1973). Reese et al. reported that the myrosinase produced by *A. sydowii* is an extracellular enzyme (Reese et al., 1958). Sakron et al. has employed a fairly simple and rapid technique to evaluate the capability of fungi to produce myrosinases using sinigrin–barium agar plates. Strains capable of producing myrosinase were identified because of formation of an opaque barium sulphate zone underneath and/or surrounding their colonies, which was further confirmed by determination of myrosinase activity in liquid cultures (Sakorn et al., 2002). Myrosinase activity was reported for the first time in a fungal pathogen of crucifers, *A. brassicicola* (Pedras and Hossain, 2011). Myrosinase activity was observed in liquid cultures and mycelial extracts of *A. brassicicola* and is part of the work described in this thesis.

The enzymatic properties of partially purified intracellular myrosinase produced by *A. niger* AKU 3302 were investigated by Ohtsuru and Hata (Ohtsuru and Hata,

1973). Maximum activity occurred at pH 6.2, optimum temperature was about 34 °C. Enzyme activity was increase by copper (I), (II), manganese (II) and cobalt (II) and was inhibited by mercury (II) and stannous (II) ions. In contrast to plant myrosinases, this enzyme was neither activated nor inhibited by L-ascorbic acid (Ohtsuru and Hata, 1973).

1.6. Conclusion

Phytoalexins and phytoanticipins (glucosinolates and derivatives) are two important components of cruciferous chemical defenses. The timing, rate of accumulation and relative amounts of phytoalexins have shown their significant role in plant defense against pathogen invasion (Dixon, 1986; Dixon and Harrison, 1994; Kuć, 1995). In vitro bioassays of phytoalexins against various fungal pathogens have shown that many phytoalexins produced in cruciferous plants are highly antifungal and thus play an important role in plant defense (Pedras et al., 2004a; Pedras and Khan, 2000; Pedras and Ahiahonu, 2002; Pedras et al., 2011a; Pedras 2011). The metabolic detoxification of phytoalexins can potentially deplete cruciferous plants from important inducible chemical defenses and render plants susceptible to pathogenic attack (Pedras et al., 2011a). It is thus important to understand the detoxification pathway of phytoalexins by phytopathogenic fungi and to inhibit these degradation processes. Both brassinin (**33**) and camalexin (**39**) are important phytoalexins. Brassinin (**33**) is not only inhibitory to many fungal pathogens but also act as a biosynthetic intermediate for many other phytoalexins (Pedras et al., 2011a). On the other hand, camalexin (**39**) has a distinct structure and also showed high antifungal activity against many cruciferous fungal pathogens (Pedras and Ahiahonu, 2005; Pedras and Ahiahonu, 2002; Pedras and

Khan, 2000). Both brassinin (**33**) and camalexin (**39**) are found to be metabolized by several cruciferous pathogens. *S. sclerotiorum*, an important pathogen of crucifers can metabolize both the compounds through glucosylation (Pedras et al, 2011). *B. cinerea* is a generalist pathogen like *S. sclerotiorum* and a very important pathogen of crucifers. Metabolism of cruciferous phytoalexins by *B. cinerea* has not been reported. Because of the close relationship between *S. sclerotiorum* and *B. cinerea* and similarity between SsBGT1 and a glucosyl transferase of *B. cinerea*, it would be very interesting to see whether *B. cinerea* metabolizes brassinin (**33**) and camalexin (**39**) as the same way of *S. sclerotiorum* or not.

The increased rate of accumulation of glucosinolates in infected or stressed plants is an indication of the role that they play in protecting plants against subsequent attacks (Agrawal et al., 2002; Mewis et al., 2005; Traw, 2002; van Dam et al., 2005). Indolyl glucosinolates, especially 4-methoxyglucobrassicin (**90**) have been claimed to play an important role in crucifer defense though the direct relationship between 4-methoxyglucobrassicin and fungal interaction has not established (Clay et al., 2009; Bednarek et al., 2009). Derivatives of glucosinolates produced due to myrosinase activity have been reported for their important biological activities. Isothiocyanates produced from glucosinolates have shown high antifungal activities against many fungal pathogens (Mithen et al., 1986; Manici et al., 1997; 2000; Bednarek et al., 2009). Metabolism of glucosinolates has been studied extensively in insects and it has been found that several glucosinolates are metabolized by cruciferous insects (Agerbirk et al., 2006; Wittstock et al, 2004; Agerbirk et al., 2010; Winde and Wittstock, 2011). The role of glucosinolates and derivatives in the interactions of crucifers with their fungal pathogens are somewhat speculative. No reports have been published about the direct interaction of these compounds with cruciferous fungal pathogens. A few studies

have shown that some *Aspergillus* species are capable of metabolizing glucosinolates using sulfatase and myrosinase (Galletti et al., 2008; Smits et al., 1993). Metabolism of glucosinolates and derivatives and also their role against cruciferous fungal pathogens are not known. Indolyl glucosinolates are structurally and biosynthetically very close relatives of cruciferous phytoalexins (Pedras et al, 2009c; Monde et al., 1995; Pedras et al., 2010). It has been found that cruciferous phytoalexins are important for defense against pathogens and it was reported that many of these phytoalexins are metabolized by plant fungal pathogens (Pedras et al., 2011a). It is important to know the ability of fungal pathogens to metabolize glucosinolates and derivatives. Such investigation can provide important information about their role in plant defense.

CHAPTER 2 : RESULTS AND DISCUSSION

2.1. Metabolism of the cruciferous phytoalexins brassinin (33) and camalexin (39) by *Botrytis cinerea*

Studying the metabolic detoxifications of phytoalexins is important to understand the interaction of plants and their pathogens. It was found that some pathogenic fungi of crucifers are able to overcome plant chemical defenses through metabolism and detoxification by utilizing a variety of enzymatic reactions (Pedras et al. 2011a). The metabolic detoxification of phytoalexins can potentially deplete cruciferous plants from important inducible chemical defenses and make plants susceptible to pathogenic attack. It is thus important to understand the detoxification pathway of phytoalexins by phytopathogenic fungi and to inhibit these degradation processes (Pedras et al., 2011b).

The detoxification pathways of phytoalexins in some cases are pathogen specific. For example, *L. maculans* oxidized brassinin (**39**) to indole-3-carboxaldehyde (**47**) and indole-3-carboxylic acid (**48**), *L. biglobosa* and *A. brassicicola* hydrolyzed brassinin (**33**) to indolyl-3-methanamine (**49**) and N_b-acetyl-3-indolylmethanamine (**50**) (Pedras et al. 2011a) whereas, *S. sclerotiorum* glucosylated brassinin (**33**) to 1-β-D-glucopyranosylbrassinin (**51**) (Figure 2.1) (Pedras and Ahiahonu, 2005). Brassinin hydrolase produced by *L. maculans* (BHLmL2) is a tetrameric protein with a molecular mass of 220 kDa and *A. brassicicola* (BHAb) is dimeric protein of 120 kDa. (Pedras et al., 2009b). Both enzymes belong to the family of amidases with the catalytic Ser/Ser/Lys triad. Serine is involved in the catalytic activity of both enzymes. Both the

BHs are functional group specific and brassinin (**33**) is the best substrate among all the compounds tested (Pedras et al. 2009b).

The gene (*SsBGT1*) responsible for transformation of brassinin by *S. sclerotiorum* was cloned and expressed in *Saccharomyces cerevisiae* and the enzyme (SsBGT1) was purified and characterized (Sexton et al., 2009). Alignment of the amino acid sequence of SsBGT1 with glucosyltransferases from other fungal species showed the closest match in *B. cinerea* with a 57% amino acid identity. This sequence similarity between *S. sclerotiorum* and *B. cinerea* is not surprising considering the phylogenetic relationship between these two species, as both are to the same family Sclerotiniaceae (Sexton et al., 2009; Hirschhaeuser and Froehlich, 2007).

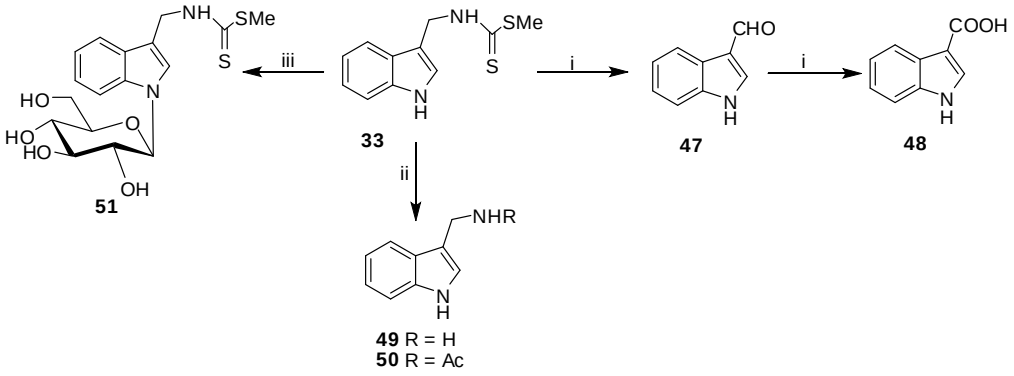


Figure 2.1 Transformation of brassinin (**33**) by: (i) *Leptosphaeria maculans*; (ii) *Leptosphaeria biglobosa*, and *Alternaria brassicicola*, and (iii) *Sclerotinia sclerotiorum*

Camalexin (**39**), another important phytoalexin from crucifers was detoxified by *R. solani* to two metabolites **53** and **54** via 5-hydroxycamalexin (**52**) (Pedras and Khan, 2000) and to 6-oxy-(O-β-D-glucopyranosyl)camalexin (**56**) via 6-hydroxy camalexin (**55**) by *S. sclerotiorum* (Figure 2.2) (Pedras and Ahiahonu, 2002).

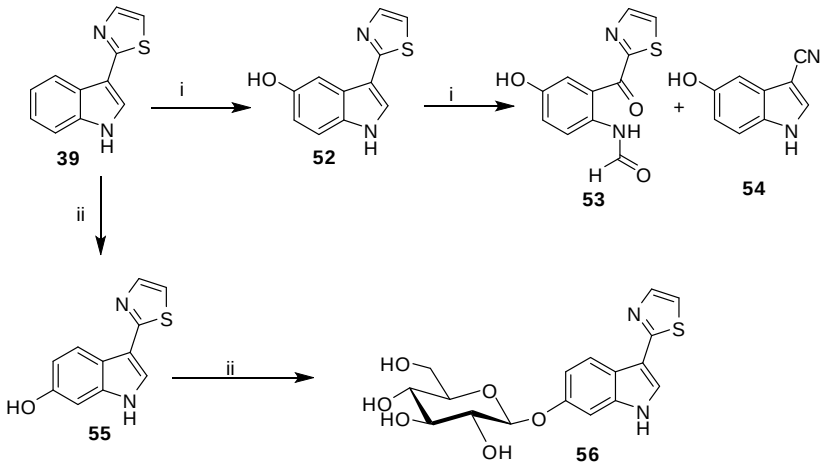


Figure 2.2 Transformation of camalexin (**39**) by: (i) *Rhizoctonia solani* and (ii) *Sclerotinia sclerotiorum*

Both brassinin (**33**) and camalexin (**39**) are very important phytoalexins with respect to cruciferous protection against some fungal pathogens. Brassinin (**33**) is not only highly inhibitory to many fungal pathogens, but also acts as a biosynthetic intermediate for many other phytoalexins; camalexin (**39**) has a distinct structure and showed high antifungal activities against many cruciferous fungal pathogens. *B. cinerea* is a generalist pathogen like *S. sclerotiorum* and a very important pathogen of crucifers. Metabolism of cruciferous phytoalexins by *B. cinerea* was not known until recently (Pedras et al., 2011b). Because of the close relationship between *S. sclerotiorum* and *B. cinerea* and similarity between SsBGT1 and a glucosyl transferase of *B. cinerea*, it was of interest to see whether *B. cinerea* metabolized brassinin (**33**) and camalexin (**39**) similar to *S. sclerotiorum*, i.e. via glucosylation.

Phytoalexins (**33** and **39**) used in this project were synthesized following known procedures (Pedras et al. 2007; Ayer et al., 1992). Before probing the biotransformation pathways of phytoalexins in *B. cinerea*, it was necessary to determine their bioactivity.

The optimum inhibitory concentrations of phytoalexins and analogues were determined using antifungal assays (Table 2.1). For biotransformation experiments, the fungus was first grown on solid media (PDA) for 2-3 days and mycelial plugs from the edge of plates were transferred to minimal media prepared as reported in the experimental and incubated for 2-3 days. Samples were added to cultures of *B. cinerea* (isolates UAMH 1784 and UAMH 1809) to obtain a final concentration of 0.10 mM, and were withdrawn from cultures immediately after addition of each compound and at different time intervals. Samples were then extracted with ethyl acetate followed by acidification of the aqueous layer and extraction with ethyl acetate and finally basification followed by extractions with CH_2Cl_2-MeOH (95:5). Each extract was carefully concentrated, dissolved in CH_3CN and analyzed by HPLC (DAD and MS detection), as described in the experimental section. Media incubated with brassinin (**33**) and camalexin (**39**) were analyzed similarly to determine the chemical stability of compounds during the incubation period.

2.1.1. Brassinin (33)

To study the metabolism of brassinin (**33**) by *B. cinerea*, brassinin (**33**) was added to the fungal culture and to media separately and samples were collected at different intervals. After extraction and concentration, samples were analyzed by HPLC (DAD and MS). Comparison of HPLC chromatograms of neutral extracts of cultures of isolates UAMH 1784 and UAMH 1809 incubated with brassinin (**33**) with that of controls showed that brassinin (**33**, t_R = 12.5 ± 0.3 min) was completely metabolized within 48 h (Figure 2.3 and Figure 2.4). The chromatograms of neutral extracts also showed the presence of additional peaks that were due to N_b-acetyl-3-

indolylmethanamine (**50**, t_R = 4.4 ± 0.2 min), 3-indolecarboxaldehyde (**47**, t_R = 5.8 ± 0.1 min) and indole-3-carboxylic acid (**48**, t_R = 3.1 ± 0.2 min). The identity of each compound was confirmed by comparing with authentic samples and quantification was carried out from respective calibration curves. The acidic extracts showed traces of brassinin (**33**) and indole-3-carboxylic acid (**48**) and the basic extracts indicated the presence of 3-indolylmethanamine (**49**).

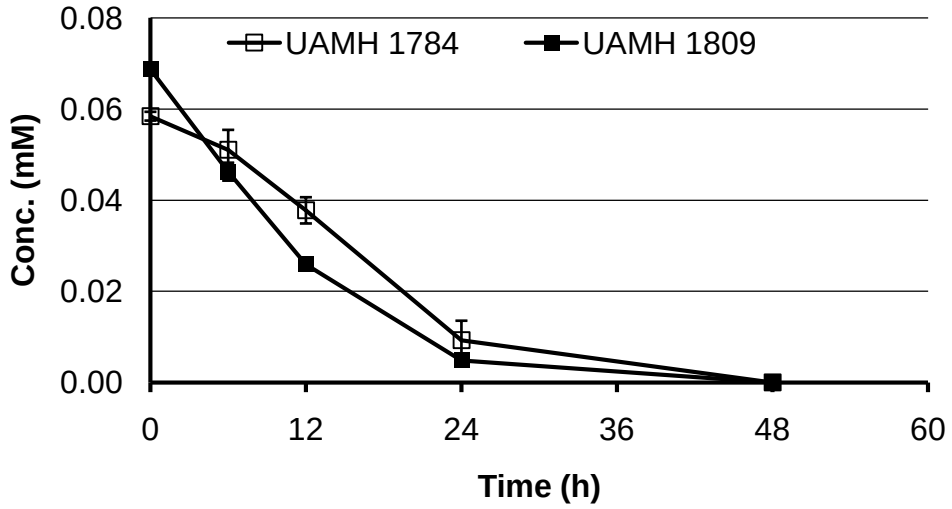


Figure 2.3 Progress curves of brassinin (**33**) transformation in cultures of *Botrytis cinerea* isolates UAMH 1784 and UAMH 1809. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

To determine the sequence of brassinin (**33**) transformation, amine (**49**) was fed to cultures of isolates of UAMH 1784 and UAMH 1809, and cultures were incubated and extracted with ethyl acetate, followed by basification of the aqueous media and extraction and finally analysis by HPLC. The HPLC chromatograms of extracts indicated that amine (**49**) was slowly transformed to N_b-acetylindolyl-3-methanamine

(**50**), 3-indolecarboxaldehyde (**47**) and indole-3-carboxylic acid (**48**). The results from individual feeding experiments confirmed that brassinin (**33**) was first hydrolyzed by *B. cinerea* to 3-indolylmethanamine (**49**), which was further metabolized to N_b-acetylindolyl-3-methanamine (**50**). N_b-Acetylindolyl-3-methanamine (**50**) was then transformed to 3-indolecarboxaldehyde (**47**) and finally to indole-3-carboxylic acid (**48**) (Figure 2.5). Compounds (**33**, **47**, **48**, **49**, and **50**) were bioassayed against *B. cinerea* and was found that these compounds (**47**, **48**, **49**, and **50**) were significantly less inhibitory to the fungus than brassinin (**33**).

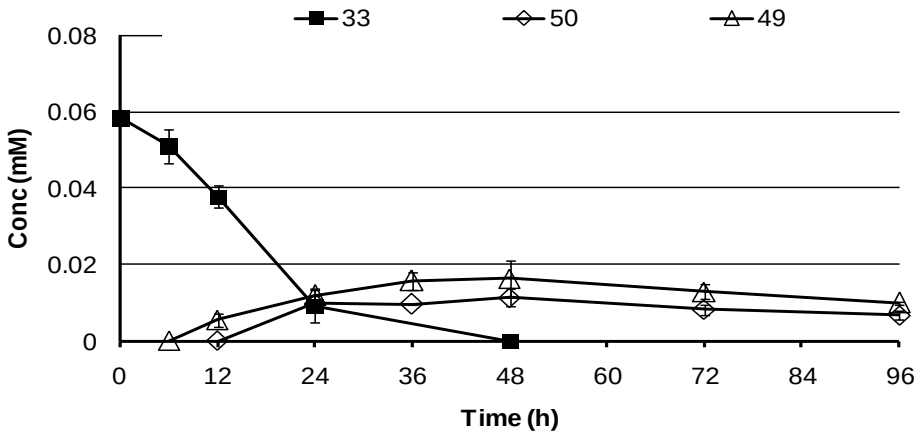


Figure 2.4 Progress curves of brassinin (**33**) transformation in cultures of *Botrytis cinerea* isolate UAMH 1784 and formation of indolyl-3-methanamine (**49**) and N_b-acetylindolyl-3-methanamine (**50**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

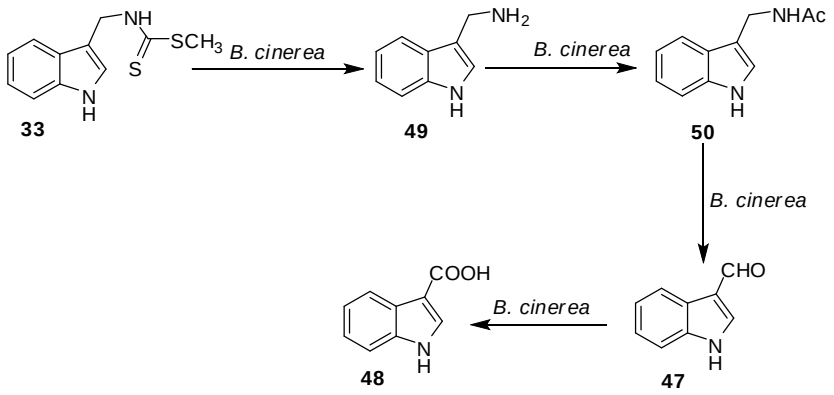


Figure 2.5 Transformation of brassinin (**33**) by *Botrtis cinerea*

2.1.2. Camalexin (39)

To study the metabolism of camalexin (**39**) by *B. cinerea*, camalexin (**39**) was dissolved in CH_3CN and added to 3-day-old cultures of the fungus to obtain a final concentration of 0.10 mM in culture medium. Samples were withdrawn from cultures immediately after addition of camalexin (**39**) and at different time intervals. After extraction and concentration, samples were analyzed by HPLC (DAD and MS). The HPLC chromatograms of neutral extracts of isolates of UAMH 1784 and UAMH 1809 incubated with camalexin (**39**) showed that it was almost completely metabolized within 12 h (Figure 2.6 and Figure 2.8). Results are the averages of at least three independent experiments.

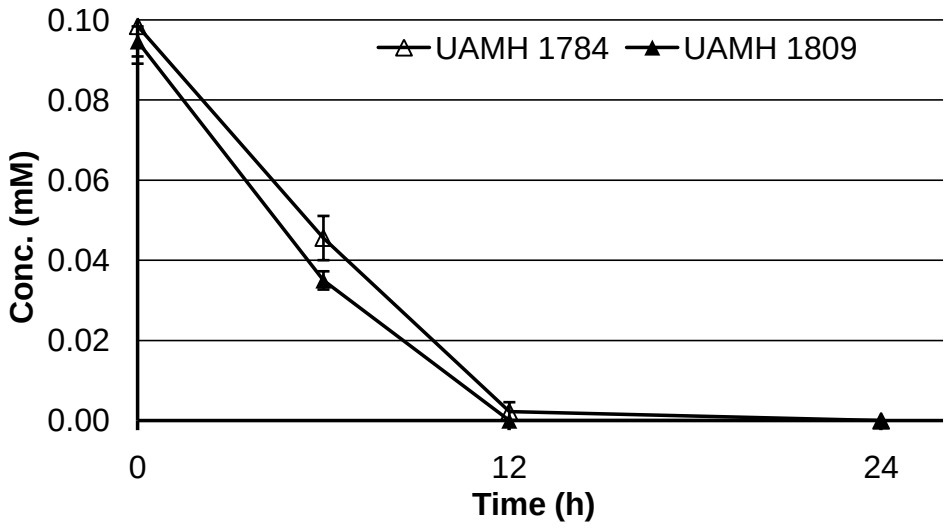


Figure 2.6 Progress curves for transformation of camalexin (**39**) by *Botrytis cinerea* isolates UAMH 1784 and UAMH 1809. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

The HPLC chromatograms of neutral extracts showed the presence of camalexin (**39**, t_R = 12.7 ± 0.1 min) and two additional peaks that were due to 3-indolecarboxynitrile (**225**, t_R = 7.8 ± 0.2 min) and indole-3-carboxylic acid (**48**, t_R = 3.6 ± 0.1 min) by comparison with the authentic samples (Figure 2.7). Two additional peaks at t_R = 4.6 and t_R = 20.5 min were observed, but their structures did not appear to be part of the metabolite libraries available in Pedras' group.

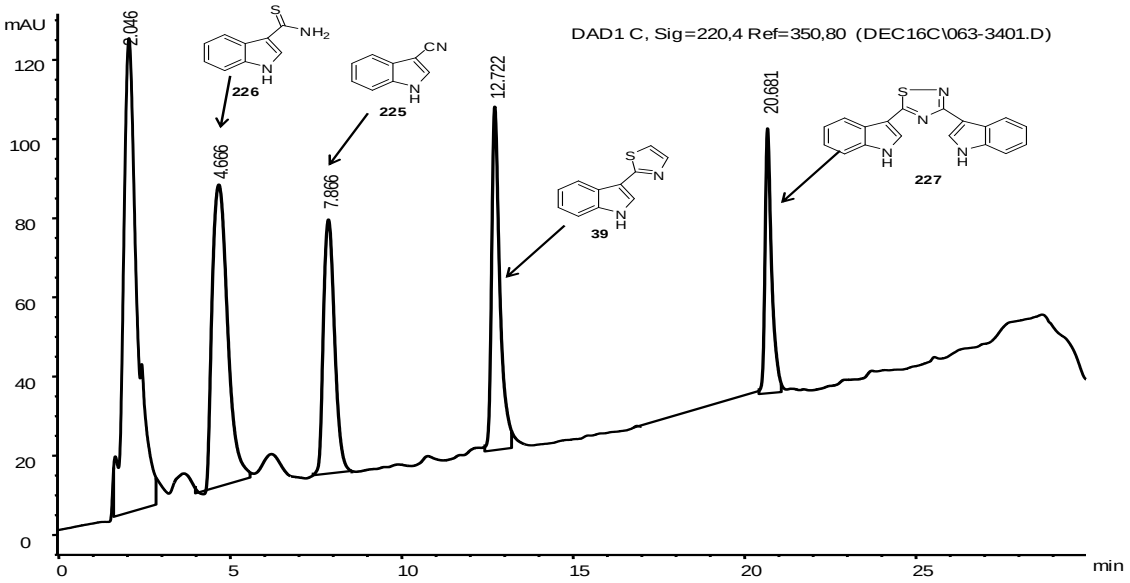


Figure 2.7 Chromatograms of neutral extracts of liquid cultures of *Botrytis cinerea* in minimal media incubated with camalexin (**39**) after 9 h.

To identify new metabolites, a larger scale culture (1L) of *B. cinerea* was incubated with camalexin (**39**) for 12 h, extracted with ethyl acetate, concentrated, and fractionated by normal phase silica gel chromatography. Each fraction was analyzed by HPLC. The fraction containing the biotransformed products, were further purified by prep. TLC. The structure of compound with t_R = 4.6 min was determined as 3-indolethiocarboxamide (**226**) by analyzing ^{1}H NMR, ^{13}C NMR and EI-HRMS spectral data. Analysis of EI-HRMS spectrum of compound with HPLC t_R = 4.6 min suggested a molecular formula $C_9H_8N_2S_2$ (calculated 176.0409, found 176.0408), whose number of protons and carbons was further confirmed by NMR spectroscopic data. The ^{1}H NMR spectrum in DMSO-d_6 of the compound revealed three broad singlets at δ 10.91, 8.08, and 7.95, which were D_2O exchangeable. A doublet of one proton at δ 7.22 with a coupling constant 3 Hz became a singlet after addition of D_2O. Two doublets at 7.75

and 6.56 ppm as well as two doublets of doublets at 6.29 and 6.26 ppm confirmed the presence of an intact indole ring with substitution at position 3. The ^{13}C NMR spectrum displayed 9 carbons, one of which was characteristic of a deshielded sp^2 carbon of a thiol group at (δ 193.6) and the remaining eight carbons were characteristic of indolyl carbons (δ 136.8 to 112.0). The presence of two D_2O exchangeable protons and thiol group suggested the existence of a thiocarbamide group attached to the indole ring at position three i.e. compound 3-indolethiocarboxamide (**226**). The structure of this compound was further confirmed by synthesis.

Due to the small amounts obtained, the structure of the compound with t_R = 20.5 min was only tentatively assigned as the bisindolylthiadiazole (**227**) from ^1H NMR and EI-HRMS data, which was later confirmed by synthesis. Analysis of HRMS-EI spectrum of compound with HPLC retention time 20.5 min suggested a molecular formula $C_{18}H_{12}N_4S$ (calc. 316.0787, found 316.0783), which number of proton and carbon was further confirmed by NMR spectroscopic data. The ^1H NMR of the compound in DMSO-d_6 showed the presence of two broad singlets at 11.26 ppm and 10.85 ppm which were D_2O exchangeable. The multiplet at 7.41ppm became two singlets upon D_2O exchange, which suggested that these two protons were coupled with the two singlets at 11.26 ppm and 10.85 ppm . The ^{13}C NMR spectrum displayed 18 carbons; 2 quaternary carbons at δ 180.2 and 169.7 ppm suggested the presence of a thiadiazole ring. Eight aromatic protons [7.61 (1H, m), 7.55 (1H, m), 6.48–6.43 (2H, m), 6.38–6.34 (2H, m)], and 16 sp^2 hybridized carbon confirmed the presence of two indolyl ring substituted at position 3. Using the thiadiazole ring as the bridge between two indole rings complete the structure of the compound as 3,5-(3,3-bisindolyl)-1,2,4-thiadiazole (**227**) which was finally confirmed by the synthesis (Pedras et al., 2011b).

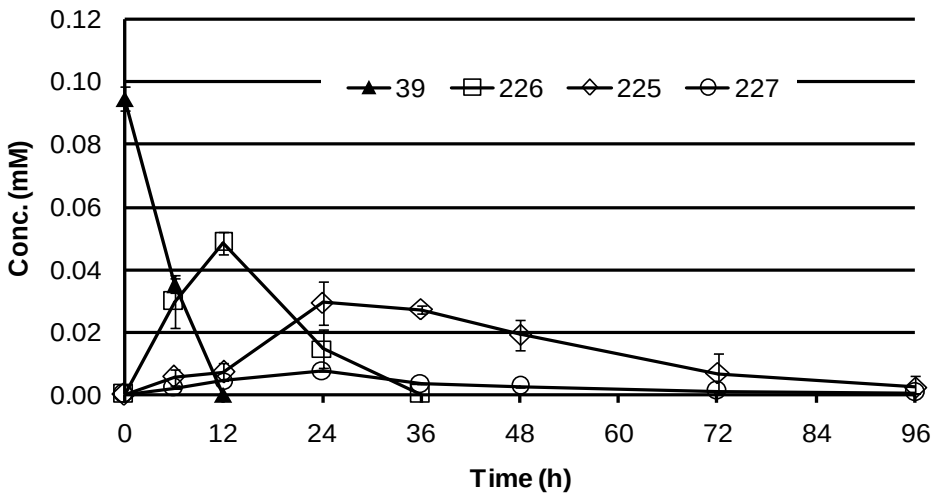


Figure 2.8 Progress curves for transformation of camalexin (**39**) by *Botrytis cinerea* isolate UAMH 1809 and formation of 3-indolethioxarboxamide (**226**), indole-3-carboxynitrile (**225**), and bisindolylthiadiazole (**227**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

To establish the sequence of steps of camalexin (**39**) transformation, the thiocarboxamide (**226**) was synthesized as described in the experimental and fed separately to cultures of isolates of UAMH 1784 and UAMH 1809. The cultures were incubated, extracted, and analyzed as by HPLC. Comparison of the HPLC chromatograms with those of control cultures showed that 3-indolethiocarboxamide (**226**) was transformed to the nitrile (**225**), which in turn was slowly transformed to indole-3-carboxylic acid (**48**) and beside this a small amount of bisindolylthiadiazole (**227**) was also detected (Figure 2.9). Analysis of the control solutions of thiocarboxamide (**226**) in media indicated that thiocarboxamide (**226**) was not stable in medium, as small amount of nitrile (**225**) and bisindolylthiadiazole (**227**) were detected in medium extracts after 6 h. However, the transformation of 3-indolethiocarboxamide (**226**) in fungal cultures ($T_{1/2}$ = 10 h and 18 h, Fig. 2.8) was much faster than in control

medium solutions ($T_{1/2}$ = 36 h, Figure 2.5), indicating that conversion of 3-indolethiocarboxamide (**226**) to 3-indolecarboxynitrile (**225**) was enzymatic. Bisindolylthiadiazole (**227**) did not form in aqueous solutions or in aqueous acetonitrile, and likely formed from a spontaneous reaction of 3-indolethiocarboxamide (**226**) to 3,5-(3′,3″-bisindolyl)-1,2,4-thiadiazole (**227**) caused by medium components (Figure 2.10).

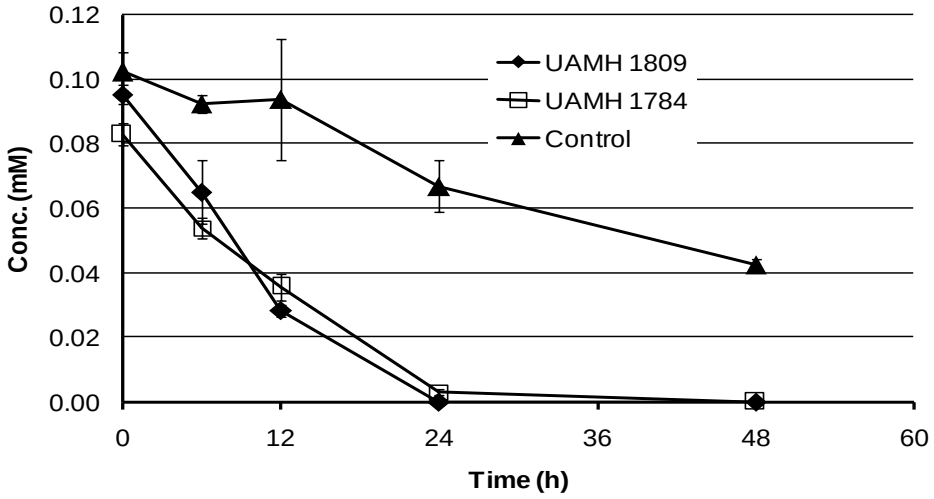


Figure 2.9 Progress curves for transformation of 3-indolethiocarboxamide (**226**) by *Botrytis cinerea* isolate UAMH 1784 and UAMH 1809. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

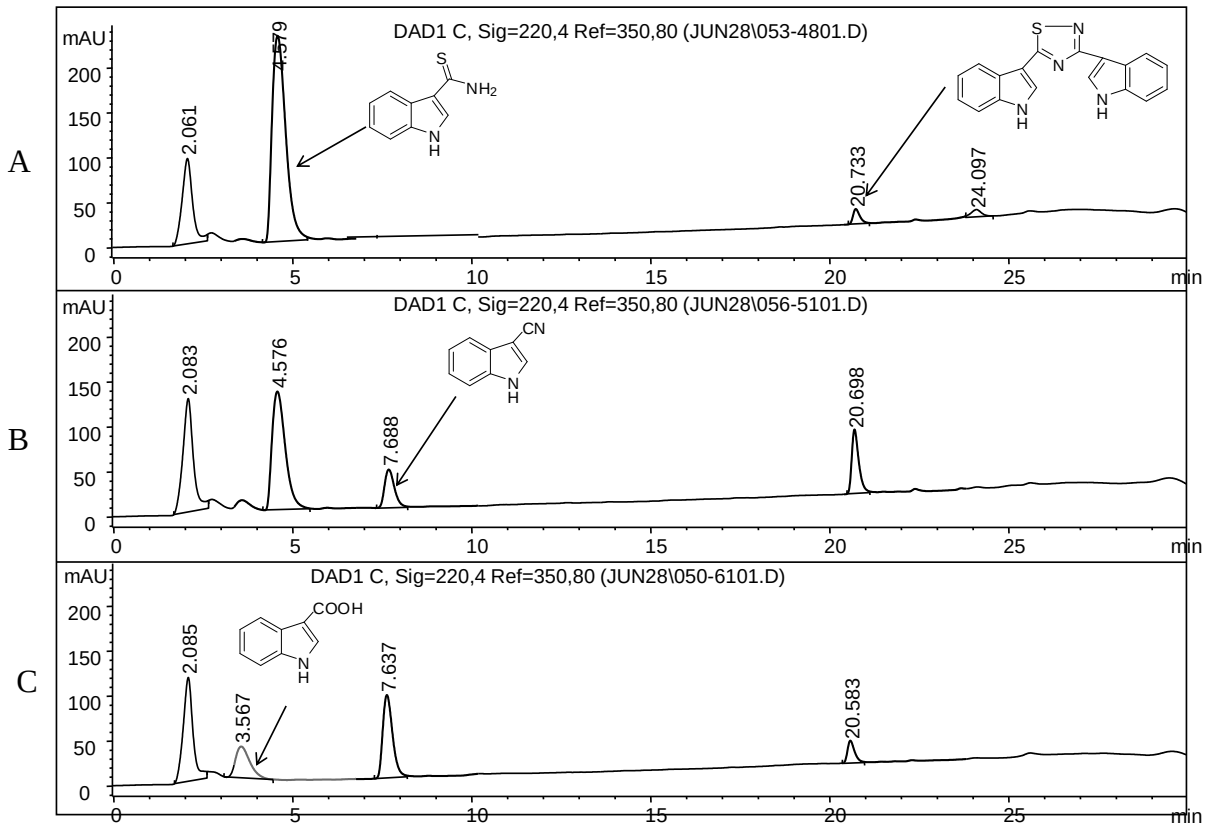


Figure 2.10 Chromatograms of neutral extracts of liquid cultures of *Botrytis cinerea* in minimal media incubated with 3-indolethiocarboxamide (**226**) after: A) 0h, B)12h, and C) 24h.

Based on the results described above the sequence of transformations of camalexin by *B. cinerea* was established as: 1) camalexin (**39**) transformed to 3-indolethiocarboxamide (**226**), 2) 3-indolethiocarboxamide (**226**) transformed to 3-indolecarboxynitrile (**225**), and finally 3) indole-3-carboxynitrile (**225**) transformed to indole-3-carboxylic acid (**48**). In addition, 3-indolethiocarboxamide (**226**) spontaneously transformed to 3,5-(3′,3″-bisindolyl)-1,2,4-thiadiazole (**227**) in medium spontaneously.

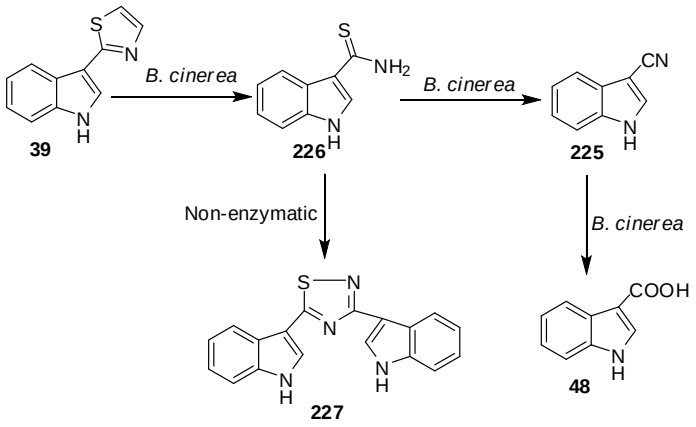


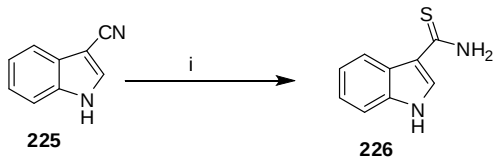
Figure 2.11 Transformation of camalexin (**39**) by *Botrytis cinerea*

2.1.3. Synthesis of metabolites

Syntheses of metabolites were very important to confirm the structures as well as study their biological activities.

3-Indolethiocarboxamide (226)

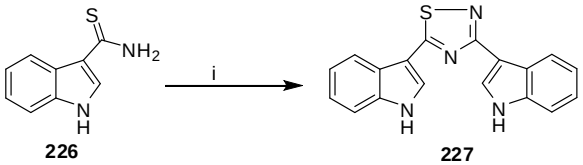
3-Indolethiocarboxamide (**226**) was synthesized by treating 3-indolecarboxynitrile (**225**) with thioacetamide in 10% HCl-DMF following a previously published procedure with a slight modification (Scheme 2.1) (Gu et al., 1999).



Scheme 2.1 Synthesis of 3-indolethiocarboxamide (**226**). Reagents and conditions: (i) CH_3CSNH_2, 10% HCl-DMF, reflux, 59%.

3,5-(3′,3″-Bisindolyl)-1,2,4-thiadiazole (227)

Synthesis of 3,5-(3′,3″-bisindolyl)-1,2-4-thiadiazole (**227**) was carried out by oxidation of 3-indolethiocarboxamide (**226**) with IBX (O-iodoxybenzoic acid) following a reported procedure for synthesizing 3,5-bisphenyl-1,2,4-thiadiazoles from thioamides in a reasonable yield (Scheme 2.2) (Patil et al., 2009).



Scheme 2.2 Synthesis of 3,5-(3′,3″-bisindolyl)-1,2,4-thiadiazole (**227**). Reagents and conditions: (i) IBX (O-iodoxybenzoic acid), CH_3CN, r.t, 45%.

2.1.4. Antifungal activity of compounds

The antifungal activity of each compound (**33**, **39**, **48**, **49**, **50**, **225**, **226**, and **227**) against *B. cinerea* was determined by employing the mycelial radial growth assays described in the experimental and results are reported in Table 2.1. Camalexin (**39**) was more inhibitory than brassinin (**33**); at 0.20 mM camalexin (**39**) inhibited completely the growth of *B. cinerea* whereas brassinin (**33**) was completely inhibitory at 0.50 mM. A slight difference in inhibition was observed when CH_3CN was used as

the solvent to dissolve the compounds instead of DMSO. Mycelial growth of the fungus was a regular circle in the presence of DMSO, but the circle was irregular in the presence of CH_3CN, which could probably be attributed to the more volatile nature of CH_3CN than DMSO.

Table 2.1 Antifungal activity of brassinin (**33**) and camalexin (**39**) (dissolved in DMSO or CH_3CN) against *Botrytis cinerea* isolates UAMH 1784 and UAMH 1809.

Phytoalexin (mM)	% Inhibition* (± S. D.)			
	UAMH 1784		UAMH 1809	
	DMSO	CH_3CN	DMSO	CH_3CN
Brassinin (**33**)				
0.50	100	100	100	100
0.20	60 (1)	51 (3)	59 (2)	60 (1)
0.10	34 (1)	22 (2)	30 (2)	31 (4)
Camalexin (**39**)				
0.20	100	100	100	100
0.10	57 (1)	38 (3)	54 (2)	38 (3)
0.05	28 (2)	32 (3)	29 (2)	28 (3)

*Percentage of growth inhibition calculated using the formula: % inhibition = 100 – [(growth on amended medium/growth in control medium) × 100]. Data are the mean ± S. D.

Antifungal activities of metabolites from brassinin (**33**) and camalexin (**39**) were determined in DMSO against isolates of *B. cinerea* UAMH 1784 and UAMH 1809. It was found that antifungal activities of metabolites of brassinin, 3-indolylmethanamine (**49**), and N_b-acetyl-3-indolylmethanamine (**50**) were significantly lower than brassinin (**33**). Similarly, the antifungal activities of metabolites of camalexin (**39**), 3-indolethiocarboxamide (**226**), 3-indolecarboxynitrile (**225**) and indole-3-carboxylic acid (**48**) were also lower than that of camalexin (**39**) (Table 2.2).

Table 2.2 Antifungal activity of metabolites of brassinin and camalexin (**48**, **49**, **50**, **225**, **226**, and **227**) (in DMSO) against *Botrytis cinerea* isolates UAMH 1784 and UAMH 1809.

Compound (mM)	% Inhibition* (± S. E.)	
	UAMH 1784	UAMH 1809
3-Indolethiocarboxamide (**226**)		
0.50	64 (2)[a]	68 (2)[a]
0.20	48 (2)	45 (2)
0.10	32 (2)	21 (2)
3-Indolecarboxynitrile (**225**)		
0.20	65 (2)[a]	60 (2)[b]
0.10	34 (2)	37 (2)
0.05	24 (2)	11 (1)
Indole-3-carboxylic acid(**48**)		
0.50	31 (2)[b]	27 (2)[c]
0.20	14 (2)	14 (2)
0.10	6 (2)	7 (1)
3,5-(3′,3″-Bisindolyl)-1,2,4-thiadiazole (**227**)		
0.50	Not soluble	Not soluble
0.20	76 (2)[c]	75 (1)[d]
0.10	68 (2)	71 (2)
0.05	49 (2)	38 (2)
3-Indolylmethanamine (**49**)		
0.50	26 (3)[d]	38 (2)[e]
0.20	19 (3)	15 (0)
0.10	14 (3)	15 (4)
N_b-Acetyl-3-indolylmethanamine (**50**)		
0.50	38 (2)[e]	30 (2)[c]
0.20	22 (2)	21 (2)
0.10	16 (2)	11 (2)

*Percentage of growth inhibition calculated using the formula: % inhibition = 100 – [(growth on amended medium/growth in control medium) x 100]. Data are the mean ± S. E.; for statistical analysis, one-way ANOVA tests were performed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column (a-e) indicate significant differences ($P < 0.05$).

2.1.5. Discussion

In conclusion, it was found that both the phytoalexins (**33** and **39**) were metabolized by isolates of *B. cinerea* UAMH 1784 and UAMH 1809. Metabolism of brassinin (**33**) to 3-indolylmethanamine (**49**) and N_b-acetyl-3-indolylmethanamine (**39**) is a detoxification pathway similar to the detoxification pathway found in *L. biglobosa* and *A. brassicicola* (Pedras et al., 2011b). On the other hand camalexin (**39**) was metabolized by *B. cinerea* to 3-indolethiocarboxamide (**226**) and finally to indole-3-carboxylic acid (**48**). In both cases, metabolites are significantly less inhibitory than corresponding phytoalexins, indicating that those transformations are detoxifications. These detoxification reactions were not expected. Despite a close similarity between SsBGT1 and a glucosyl transferase in *B. cinerea* with a 57% amino acid identity, none of the phytoalexins was glucosylated (Pedras et al. 2011b).

2.2. Metabolism of aryl and indolyl glucosinolates (65, 66, 86, 87, and 90) and derivatives (40, 102, 103, 107, 110, 113, 159, 170, 185, 202, 228, and 233) by plant pathogenic fungi

Metabolic studies of indole glucosinolates and derivatives are very important as they have been reported playing important roles in cruciferous chemical defenses (Fahey et al., 2001; Keck and Finely, 2004; Fenwick and Heaney, 1983; Fahey et al., 2001; Traka and Mithen, 2009; Vig et al., 2009; Hopkins et al., 2009). No report has been published about the direct interaction of indole glucosinolates with cruciferous fungal pathogens until recently (Pedras and Hossain, 2011). Since some cruciferous phytoalexins are metabolized and detoxified by several cruciferous pathogens, it was important to know whether indole glucosinolates are metabolized. Indolyl

glucosinolates and derivatives were the centre of this study because of their close biological relationship with cruciferous phytoalexins and their production is induced by stress in many cruciferous plants. It was also of interest to compare the metabolism of indolyl and aryl glucosinolates carried out by different fungal pathogens (specialists and generalists). Toward this end, two generalist pathogens (*R. solani* and *S. sclerotiorum*), and one specialist pathogen of crucifers (*A. brassicicola*) were used. In addition, three indolyl glucosinolates (glucobrassicin (**86**), 1-methoxyglucobrassicin (**87**), and 4-methoxyglucobrassicin (**90**)), two aryl glucosinolates (phenyl glucosinolate (**65**) and benzylglucosinolate (**66**)) and their stable derivatives were selected for metabolic studies.

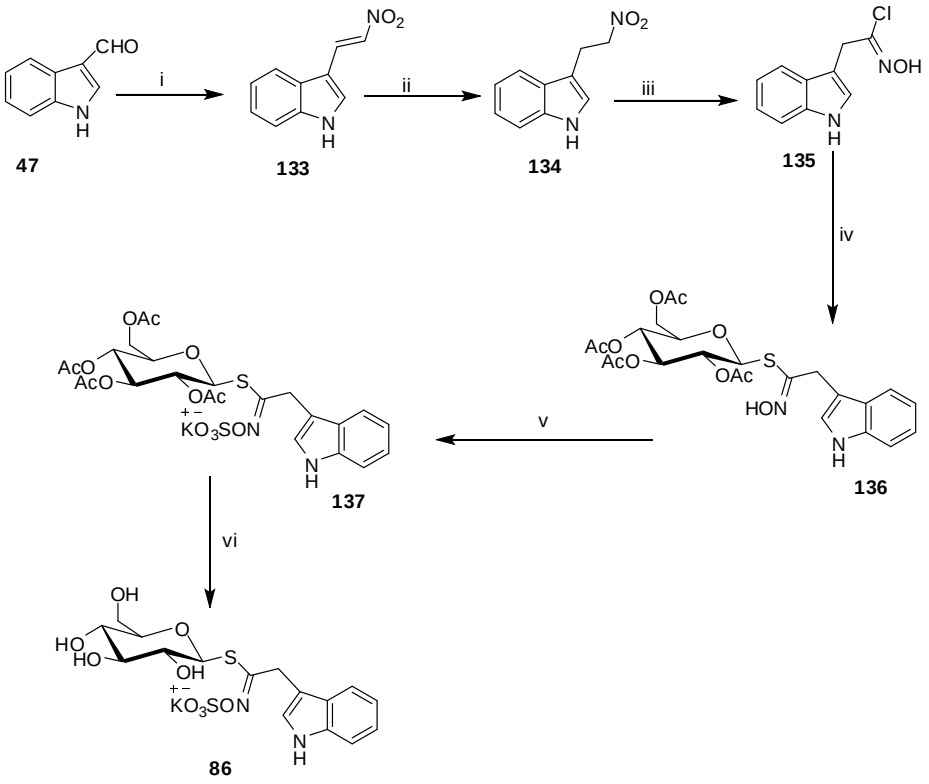
2.2.1. Chemical synthesis of compounds

Synthesis of glucosinolates in sufficient amount was one of the major challenges of this project. Though syntheses of several aliphatic (methyl, 2-propenyl etc.) and aryl (benzyl, phenylethyl etc.) glucosinolates were reported in the early nineteen sixties (Fahey et al., 2001; Rollin and Tatibouëtt, 2011), synthesis of these compounds afforded very low yields. The first synthesis of an indole glucosinolate (glucobrassicin (**86**)) was reported by Viaud and Rollin with an overall yield of ca. 10% (method A) (Viaud and Rollin, 1990). An alternative synthetic route was explored by the same group several years later with similar overall yield (method B) (Cassel et al. 1998). In this project synthesis of indolyl glucosinolates and desulfoglucosinolates were carried out by a slight modification of method A (scheme 2.3) as method B (scheme 2.4) was not found suitable for the methoxy derivatives (1-methoxy glucobrassicin (**87**) and 4-methoxy glucobrassicin (**90**)).

The first synthesis of an aryl glucosinolate was reported by Ettlinger and Lundeen (1957), who described the synthesis of benzylglucosinolate (**66**) through phenylacethiohydroxamic acid (**117**) starting from benzyl chloride (**116**) with an overall yield of 7% (Scheme 1.1). An alternative synthetic route was reported by Benn (1964) for aliphatic and aromatic glucosinolates via aldoxime. Chlorination of aldoximes using chlorine gas afforded the corresponding hydroximoyl chlorides, which were then used as crude intermediates to couple with tetra-O-acetylated thioglucopyranose (**119**) to produce a glucosylated thiohydroximates. Subsequent O-sulfation with sulfur trioxide pyridine complex followed by treatment with $KHCO_3$ resulted in the formation of tetraacetylated glucosinolate, which was then hydrolyzed with a suitable base to deliver the expected glucosinolate in much better yield compared to the earlier method. Mays et al. reported the synthesis of several non-natural glucosinolates from oxime using NCS as the chlorinating agent and NaOMe as the base for deacetylation/hydrolysis with an excellent overall yield (87 – 89%) (Mays et al., 2008). In this project, syntheses of phenylglucosinolate (**65**) and benzylglucosinolate (**66**) were carried out following the procedure reported by Mays et al. Phenyldesulfoglucosinolate (**228**) and benzyldesulfoglucosinolate (**202**) was synthesized for the first time following the same procedure. Compounds **103** (Pedras et al., 2007), **107** (Piironen and Virtanen, 1962), **113** (Jackson et al., 1987), **159** and **233** (Robertson and Botting, 1999) were synthesized according to literature procedures. Commercially available compounds (**40**, **110**, **170**, **185**, **234**, and **235**) were used as such, after confirming their purity by HPLC analysis.

Glucobrassicin (86) (method A)

Glucobrassicin (**86**) was synthesized as previously published (method A) with slight modifications as per Scheme 2.3 (Viaud and Rollin, 1990). 3-Indolecarboxaldehyde (**47**) was allowed to react with nitromethane and ammonium acetate to give 3´-(1-nitrovinyl)indole (**133**), which was then reduced with $NaBH_4$ in the presence of the silica gel, chloroform and isopropyl alcohol to produce 3´-(1-nitroethyl)indole (**134**). 3´-(1-Nitroethyl)indole **(134)** was then treated with sodium methoxide to form the corresponding nitronoate, which was then treated with $SOCl_2$ to yield indolyl-3- acetohyhydroximoylchloride (**135**) at -40 °C. Using Sodium hydride (NaH) instead of NaOMe at 0 ° C was tried. The yield of the reaction was similar in both cases (ca. 36%) but the results were not consistent for the later procedure. The crude intermediate produced from chlorination was coupled with thioglucose to produce indolyl-3-tetraacetylglucoacetothiohydroximate (**136**), which was then treated with chlorosulfonic acid and $KHCO_3$ to form tetraacetyl glucobrassicin (**137**). Finally, **137** was hydrolyzed with potassium methoxide to produce glucobrassicin (**86**) in 11 % overall yield. The most sensitive step of this synthesis was the conversion of nitroethyl indole (**134**) to hydroximoyl chloride (**135**) and then coupling of **135** with thioglucose to form the tetraacetyl glucobrassicin thiohydroximate (**136**). The reaction was performed at -40 °C under argon to avoid decomposition of **135**. Hydroximoyl chloride was reported as unstable, thus it was not purified and was used immediately into the next step. The overall yield over these two steps was consistent, ca. 40 %.



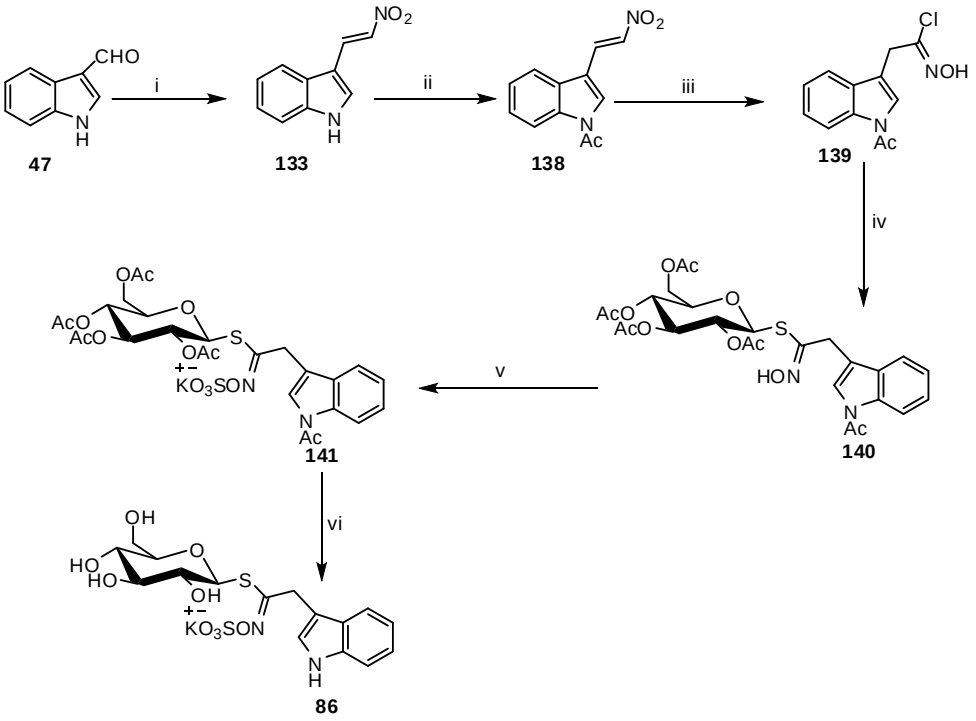
Scheme 2.3 Synthesis of glucobrassicin (**86**). Reagents: (i) CH_3NO_2, ammonium acetate, 120 -130º C, 65%; (ii) $NaBH_4$, SiO_2, $CHCl_3$, i-PrOH, 55%; (iii) MeONa, MeOH, $SOCl_2$, DME, ; (iv) Thioglucose, Et_3N, Et_2O/CH_2Cl_2, 41%; (v) HSO_3Cl, pyridine, 66%; (vi) MeOK, MeOH, quantitative (Viaud and Rollin, 1990).

Reduction of nitrovinylindole (**133**) was found to be better (ca. 10%) when the compound was reduced by $NaBH_4$ in CH_3OH and THF but it was not reproducible for 1-methoxy and 4-methoxynitrovinylindole derivatives. In the final step, deacetylation of tetraacetyl glucobrassicin (**137**) was also found to be time sensitive, especially for 1-methoxy (**86**) and 4-methoxy glucobrassicin (**90**). Only 2 h reaction time was enough in contrast to 24 h reported in the original procedure, longer reaction time resulted in formation of a mixture of inseparable compounds.

Glucobrassicin (86) (method B)

Glucobrassicin (**86**) was also synthesized following the more recent (method B) as per Scheme 2.4. 3-Indolecarboxaldehyde (**47**) was allowed to react with nitromethane and ammonium acetate to form 3´-(1-nitrovinyl)indole (**133**) which was then acetylated with acetic anhydride to give N-acetyl-3-(2´-nitrovinyl)indole (**138**). N-Acetyl-3´-(1-nitrovinyl)indole (**138**) was treated with $TiCl_4$ and triethylsilane to yield N-acetyl indolyl-3-acetohyhydroximoylchloride (**139**). The crude intermediate was coupled with thioglucose and triethyl amine to produce N-acetylindolyl-3-tetra-O-acetylglucoacetothiohydroximate (**140**) which was then treated with chlorosulfonic acid and $KHCO_3$ to form N-acetyltetra-O-acetyl glucobrassicin (**141**). Finally, **141** was hydrolyzed with potassium methoxide to give glucobrassicin (**86**) in 14 % overall yield (Cassel et al., 1998).

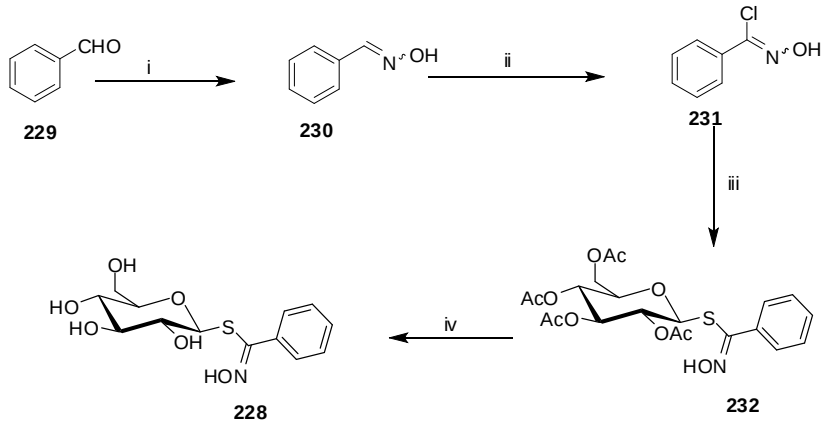
The overall yield of method B was slightly better than method A (14% vs. 11%) and also used fewer steps than method A, but as mentioned earlier this synthetic route was not suitable for methoxy derivatives (1-methoxy and 4-methoxy glucobrassicin; **87** and **90**). In both cases, several products were formed when N-acetyl nitrovinylindole was treated with $TiCl_4$ followed by coupling with thioglucose.



Scheme 2.4 Synthesis of glucobrassicin (**86**). Reagents: (i) CH_3NO_2, ammonium acetate, 120 -130º C, 65%; (ii) Ac_2O, pyridine, 92%; (iii) $TiCl_4$, Et_3SiH, CH_2Cl_2,; (iv) Thioglucose, Et_3N, Et_2O/CH_2Cl_2, 36%; (v) HSO_3Cl, pyridine, $KHCO_3$, 64%; (vi) MeOK, MeOH, quantitative (Cassel et al., 1998).

Phenyldesulfoglucosinolate (228)

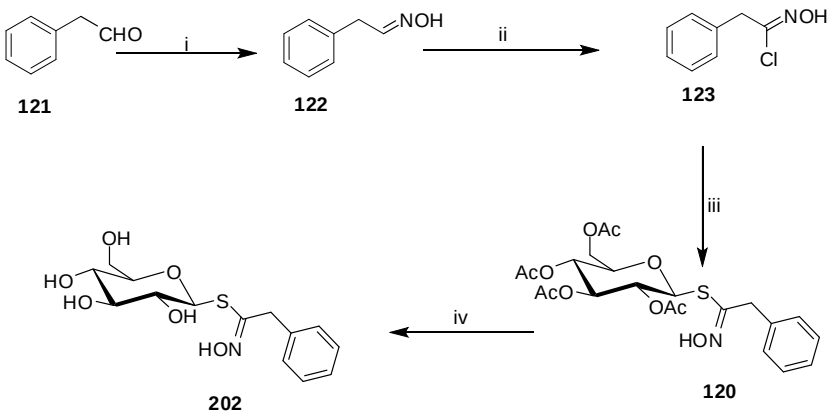
Benzaldehyde (**229**) was allowed to react with hydroxylamine hydrochloride and sodium carbonate to give benzaldehyde oxime (**230**). Benzaldehyde oxime (**230**) was then treated with N-chlorosuccinimide to form the corresponding hydroximoyl chloride (**231**). The crude intermediate was coupled with thioglucose and triethyl amine to produce the corresponding thiohydroximate (**232**), which was then hydrolyzed with potassium methoxide to produce phenyldesulfoglucosinolate (**229**) in 46 % overall yield (Scheme 2.5).



Scheme 2.5 Synthesis of phenyldesulfoglucosinolate (**228**). Reagents: i. $NH_2OH.HCl$, Na_2CO_3, H_2O, EtOH, 90%; ii. NCS, Pyridine, DMF; iii. Thioglucose, Et_3N, 60%; iv. MeOK, MeOH, 85% (Mays et al., 2008).

Benzyldesulfoglucosinolate (202)

Phenyl acetaldehyde (**121**) was allowed to react with hydroxylamine hydrochloride and sodium carbonate to give phenyl acetaldehyde oxime (**122**). Phenyl acetaldehyde oxime (**122**) was then treated with N-chlorosuccinimide to form the corresponding hydroximoyl chloride (**123**). The crude intermediate was coupled with thioglucose to produce the corresponding thiohydroximate (**120**), which was then was hydrolyzed with potassium methoxide to afford benzyldesulfoglucosinolate (**202**) in 55% overall yield (Scheme 2.6).



Scheme 2.6 Synthesis of benzyldesulfoglucosinolate (**202**). Reagents: i. $NH_2OH.HCl$, Na_2CO_3, H_2O, EtOH, 70%; ii. NCS, Pyridine, DMF; iii. Thioglucose, Et_3N, 78%; iv. MeOK, MeOH, 100%.

2.2.2. Antifungal activity of compounds

Glucosinolates (**65**-**66**, **86**-**87**, and **90**) desulfoglucosinolates (**159**, **202**, **233**, and **228**), and derivatives of glucosinolates, isothiocyanates (**170** and **234**), nitriles (**40**, **102**, **103**, **185**, and **235**), ascorbigen (**107**), indolyl-3-methanol (**110**), and diindolyl-3-methane (**113**), were assayed for antifungal activity against *A. brassicicola*, *R. solani* and *S. sclerotiorum* (summarized in Table 2.3 and Table 2.4). For glucosinolates and desulfoglucosinolates, H_2O was used as the solvent and for derivatives, DMSO was used as the solvent. In case of benzyl isothiocyanate and phenyl isothiocyanate CH_3CN was used as the solvent instead of DMSO. When DMSO was used, no inhibition was observed against any pathogen.

Table 2.3 Antifungal activity of glucosinolates (**65-66**, **86-87**, and **90**) and desulfoglucosinolates (**159**, **202**, **228** and **233**) against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light.

Compound name	Conc. (mM)	% Inhibition		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
Glucobrassicin (**86**)	0.50	NI	NI	NI
1-Methoxyglucobrassicin (**87**)	0.50	NI	NI	NI
4-Methoxyglucobrassicin (**90**)	0.50	NI	NI	NI
Phenyl glucosinolate (**65**)	0.50	NI	NI	NI
Benzylglucosinolate(**66**)	0.50	NI	NI	NI
Desulfoglucobrassicin (**159**)	0.50	NI	NI	NI
1-Methoxydesulfo glucobrassicin (**233**)	0.50	NI	NI	NI
Phenyl desulfo glucosinolate (**228**)	0.50	NI	NI	NI
Benzyl desulfo glucosinolate (**202**)	0.50	NI	NI	NI

Table 2.4 Antifungal activity of glucosinolate derivatives (**40**, **102**, **103**, **107**, **110**, **113**, **170**, **185**, **234**, and **235**) against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light.

Compound name	Conc. (mM)	% Inhibition (± S. E.)*		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
Indolyl-3-acetonitrile (**40**)	0.50	27 (4)^{a,x}	41 (3)^{a,y}	27 (0)^{a,x}
	0.20	13(2)	15 (3)	16 (1)
	0.10	3 (1)	3 (3)	4 (1)
1-Methoxyindolyl-3-acetonitrile (**102**)	0.50	43 (2)^{b,x}	100 (0)^{b,y}	54 (0)^{b,z}
	0.20	29 (0)	45 (2)	13 (2)
	0.10	18 (0)	29 (2)	0 (0)

Compound name	Conc. (mM)	% Inhibition (± S. E.)*		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
4-Methoxyindolyl-3-acetonitrile (**103**)	0.50	59 (2)^{c,x}	70 (2)^{c,y}	77 (0)^{c,y}
	0.20	15 (2)	46 (0)	46 (0)
	0.10	0 (0)	26 (2)	24 (2)
Ascorbigen (**107**)	0.50	36 (2)^{d,x}	12 (1)^{d,y}	10 (0)^{d,y}
	0.20	12 (0)	6 (0)	7 (0)
	0.10	0 (0)	0 (0)	3 (0)
Indolyl-3-methanol (**110**)	0.50	43 (2)^{b,x}	44 (0)^{b,x}	73 (1)^{c,y}
	0.20	29 (0)	27 (1)	60 (0)
	0.10	18 (0)	11 (0)	41 (1)
Diindolyl-3-methane (**113**)	0.50	100 (0)^{e,x}	100 (0)^{b,x}	100 (0)^{e,x}
	0.20	77 (2)	61 (2)	81 (3)
	0.10	60 (2)	32 (2)	72 (3)
Benzyl isothiocyanate (**170**)	0.50	11 (0.5)^{f,x}	100 (0)^{b,y}	100 (0)^{e,y}
	0.20	0	59 (0.5)	50 (0.4)
	0.10	0	20 (0.5)	18 (0.6)
Phenyl isothiocyanate (**234**)	0.50	0^{g,x}	44 (4)^{a,y}	63 (0.3)^{f,z}
	0.20	0	16 (0.4)	48 (0.6)
	0.10	0	9 (0.8)	10 (0.5)
Phenyl acetonitrile (**185**)	0.50	0^{g,x}	51 (0.6)^{a,y}	59 (0.6)^{f,z}
	0.20	0	29 (1)	44 (0.5)
	0.10	0	9 (0.7)	17 (0.8)
Benzonitrile (**235**)	0.50	0^{g,x}	51 (0.6)^{e,y}	59 (0.6)^{g,z}
	0.20	0	29 (1)	44 (0.5)
	0.10	0	9 (0.7)	17 (0.8)

*Percentage of growth inhibition calculated using the formula: % inhibition = 100 – [(growth on amended medium/growth in control medium) x 100]. Data are the mean ± S. E.; for statistical analysis, one-way ANOVA tests were performed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column (a-g) and in the same row (x-z) indicate significant differences ($P < 0.05$).

2.2.3. Analysis of samples

Biotransformation studies of glucosinolates and derivatives were carried out in sterile H_2O. As most of these compounds are highly polar it was not possible to extract these compounds from fungal cultures using organic solvents. The fungus (*A. brassicicola*, *R. solani,* or *S. sclerotiorum*) was first incubated in minimal media for 2-3 days and after that fungal mycelium was filtered, washed, and finally transferred into sterile water as described in the experimental. Liquid cultures of each fungus were then incubated with compounds for various time periods, samples were withdrawn from cultures immediately after addition of each compound and up to five days, immediately frozen and then lyophilized (Pedras and Suchy, 2005). Media incubated with each compound (control solutions) were treated similarly to determine the chemical stability of compounds during the incubation experiments. The lyophilized compounds were then dissolved in CH_3OH: H_2O (1:1) and analyzed by HPLC (DAD and ESI).

Several techniques have been used to analyze glucosinolates, including gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Among these techniques GC was found to be suitable for analysis of low molecular weight volatile derivatives of glucosinolates, whereas HPLC was found better for intact glucosinolates albeit with several difficulties. Gas chromatography was the earliest technique used to identify and quantify individual glucosinolates, desulfoglucosinolates and derivatives. Less polar derivatives were extracted by dichloromethane and analyzed by GLC. In case of glucosinolates and desulfoglucosinolates it was necessary to make the compounds volatile by derivatisation. Isothermal GC was not successful to separate indolyl glucosinolates. The major drawback of GC method was the thermal instability of many of those compounds but still useful to analyze many volatile compounds produced from

glucosinolates due to myrosinase activity, especially for low molecular isothiocyanates and nitriles (Fahey et al. 2001).

The ionic nature of glucosinolates makes them very difficult to analyze by HPLC. Glucosinolates were analyzed by converting them to desulfoglucosinolates usually on small ion-exchange columns. Use of HPLC for the separation of desulfoglucosinolates on a reverse phase C_{18} column without using buffer or ion-paring reagent was first described by Minchinton et al. (1982). This method though effective requires a complicated sample preparation and is time consuming. An enzyme sulfatase sometimes used to reduce the time of desulfation (Landerouin et al., 1987; Quinsac and Ribaillier, 1987). Reverse-phase HPLC with ion pairing has been used alternatively for the separation and identification of intact glucosinolates (Helboe et al., 1980). Tetrabutyl ammonium sulfate as an ion-pairing reagent was used with C18 solid-phase separation by Betz and Page (1990) to separate several aliphatic and aromatic glucosinolates. Formic acid and triethyl amine (TEA) were used with a reverse phase ion pair HPLC method followed by negative-ion ESI mass spectroscopy by Zrybko et al. (1997) to identify and quantify glucosinolates and derivatives successfully. For this project, a reverse-phase HPLC-DAD detection method containing formic acid was used to identify and quantify glucosinolates and desulfoglucosinolates.

Sources of variations in results

The possible sources of variations and errors included in the sample analysis (quantification of glucosinolates and derivatives) are as follows:

a) Errors in weighing of sample and making stock solutions: the last decimal place of the balance is uncertain and can cause substantial error due to weighing (up to 4% for 1 mg of sample).

b) Errors in measuring the volume of samples transferred into fungal cultures.

c) Errors due to measuring the volume of samples collected for lyophilization.

d) Variations due to the biological system: the major source of variation usually come from the fungal cultures. The growth of fungus is not identical in every culture; this can lead to different rates of metabolism. It is estimated that the variations due to different growth can amount to 10-15%.

f) Instrumental errors: sources of error in an HPLC are mainly due the injector, which can be maximum 2%.

2.2.4. Metabolism of glucosinolates

Biotransformation of glucobrassicin (**86**), 1-methoxyglucobrassicin (**87**), 4-methoxyglucobrassicin (**90**) (Pedras and Hossain, 2011), phenylglucosinolate (**65**) and benzylglucosinolate (**66**) have been studied in the cultures of the cruciferous fungal pathoges *A. brassicicola, R. solani* and *S. sclerotiorum*. Glucosinolates were dissolved in water and then added to fungal cultures of *A. brassicicola, R. solani*, and *S. sclerotiorum* in sterile water to get a final concentration of 0.10 mM. Control samples were also monitored every time by adding glucosinolates in sterile water. Equal amount of samples were collected from fungal cultures as well as from control at 0h and at different time intervals, immediately frozen and lyophilized, dissolved in CH_3OH:H_2O (1:1) to analyze by HPLC.

Glucobrassicin (86)

HPLC analysis of concentrated culture samples indicated no transformation of glucobrassicin (**86**) by *A. brassicicola*, *R. solani* and *S. sclerotiorum* (Figure 2.12). The compound was found to be stable in fungal cultures and in H_2O over 5 days.

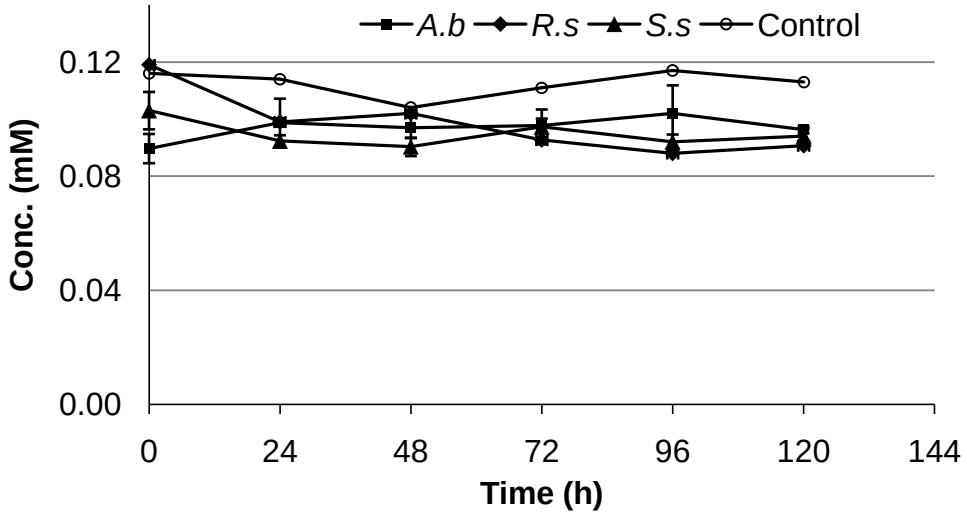


Figure 2.12 Progress curves of glucobrassicin (**86**) in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviation.

1-Methoxyglucobrassicin (87)

HPLC analysis of concentrated culture samples indicated no transformation of **87** by *A. brassicicola*, *R. solani*, or by *S. sclerotiorum* (Figure 2.13). 1-Methoxy glucobrassicin (**87**) was less stable in H_2O compared to two other glucobrassicins tested. A slow decrease in concentration of **87** was observed in fungal cultures and in H_2O.

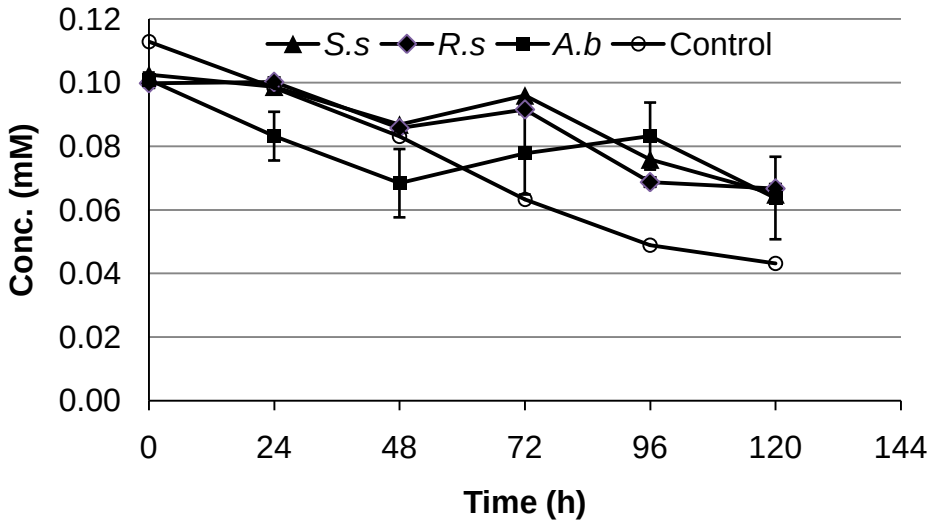


Figure 2.13 Progress curves of 1-methoxyglucobrassicin (**87**) in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

4-Methoxyglucobrassicin (90)

HPLC analysis of concentrated culture samples indicated no transformation of 4-methoxyglucobrassicin (**90**) by *A. brassicicola, R. solani* or by *S. sclerotiorum* (Figure 2.14). Similar to glucobrassicin (**86**), **90** was also found to be stable in fungal cultures and in H_2O over 5 days.

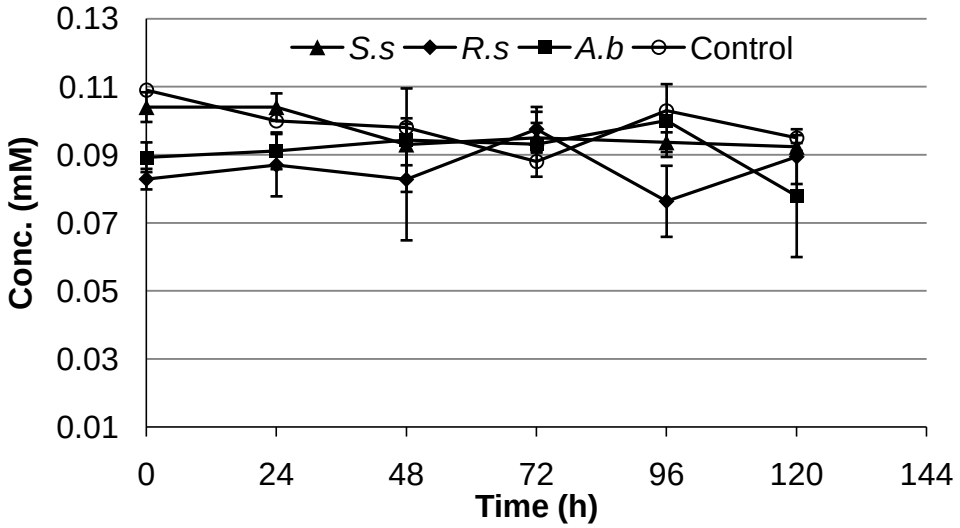


Figure 2.14 Progress curve of 4-methoxyglucobrassicin (**90**) in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

Phenylglucosinolate (65)

HPLC analysis of concentrated culture samples indicated transformation of phenyl glucosinolate (**65**) by *A. brassicicola* to benzonitrile (**235**) (100% metabolized in 72 h) (Figure 2.16) (**24**) but no transformation was observed in cultures of *R. solani* and *S. sclerotiorum* up to 120h (Figure 2.15). The identity of benzonitrile (**235**) was confirmed by comparing HPLC retention time and UV spectra with commercially available compound.

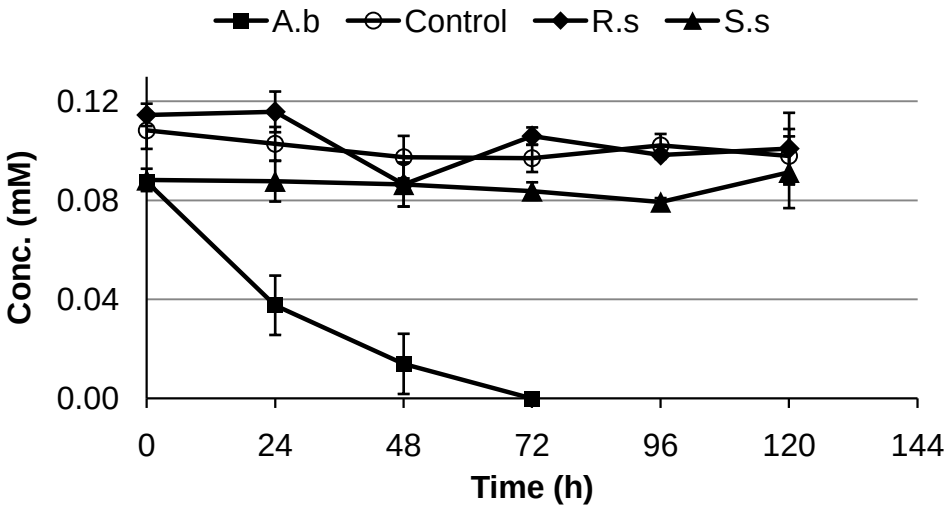


Figure 2.15 Progress curves of phenylglucosinolate (**235**) transformation in cultures of *Alternaria brassicicola, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

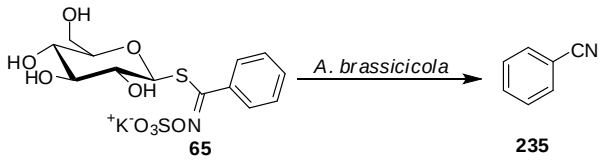


Figure 2.16 Transformation of phenyl glucosinolate (**65**) by *Alternaria brassicicola*

Benzylglucosinolate (66)

HPLC analysis of concentrated culture samples indicated transformation of benzylglucosinolate (**66**) by *A. brassicicola* to phenyl acetonitrile (**185**) in 24 h, which was further metabolized to phenyl acetic acid (**199**), a traces of benzyl isothiocyanate (**170**) was detected in the culture also (Figure 2.18) but no transformation was observed in cultures of *R. solani* and *S. sclerotiorum* (Figure 2.17). The identities of metabolites

were confirmed by comparing HPLC retention time and UV spectra with commercially available compounds.

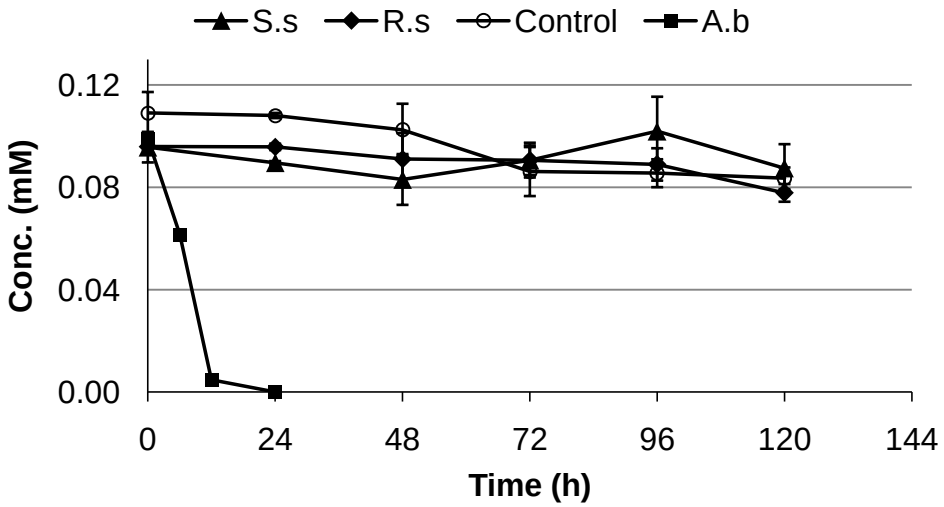


Figure 2.17 Progress curve of benzylglucosinolate (**66**) transformation in cultures of *Alternaria brassicicola, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

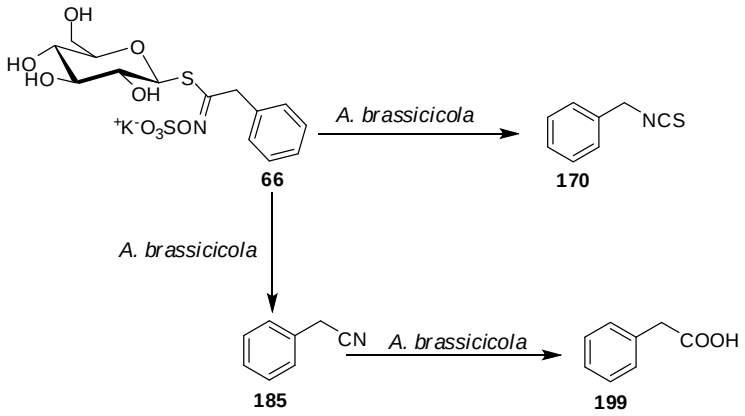


Figure 2.18 Transformation of benzylglucosinolate (**66**) by *Alternaria brassicicola*

2.2.5. Metabolism of desulfoglucosinolates (**159**, **202**, **228**, and **233)**

Metabolism of desulfoglucobrassicin (**159**), 1-methoxydesulfoglucobrassicin (**233**) (Pedras and Hossain, 2011), benzyldesulfoglucosinolate (**202**), and phenyldesulfoglucosinolate (**228**) was studied by *A. brassicicola*, *R. solani*, and *S. sclerotiorum*. Similar to glucosinolates desulfoglucosinolates were dissolved in water and then added to fungal cultures of *A. brassicicola*, *R. solani*, and *S. sclerotiorum* in sterile water to get a final concentration of 0.10 mM. Control samples were also monitored every time by desulfoglucosinolates in sterile water. Equal amount of samples were collected from fungal cultures as well as from control at 0h and at different time intervals, immediately frozen and lyophilized, dissolved in CH_3OH:H_2O (1:1) to analyze by HPLC.

Desulfoglucobrassicin (159)

Desulfoglucobrassicin (**159**) was found to be transformed by both *R. solani* and S. *sclerotiorum* to indoly-3-acetonitrile (**40**) which was eventually transformed to indolyl-3-carboxylic acid (**236**) (Figure 2.21). The transformation of desulfoglucobrassicin (**159**) to nitrile (**40**) by *S. sclerotiorum* ($t_{1/2}$ = 108 h) is slower than that by *R. solani* ($t_{1/2}$ = 24 h) (Fig 2.19 and 2.20) however, *A. brassicicola* did not metabolize **159** (Figure 2.22) (Pedras and Hossain, 2011).

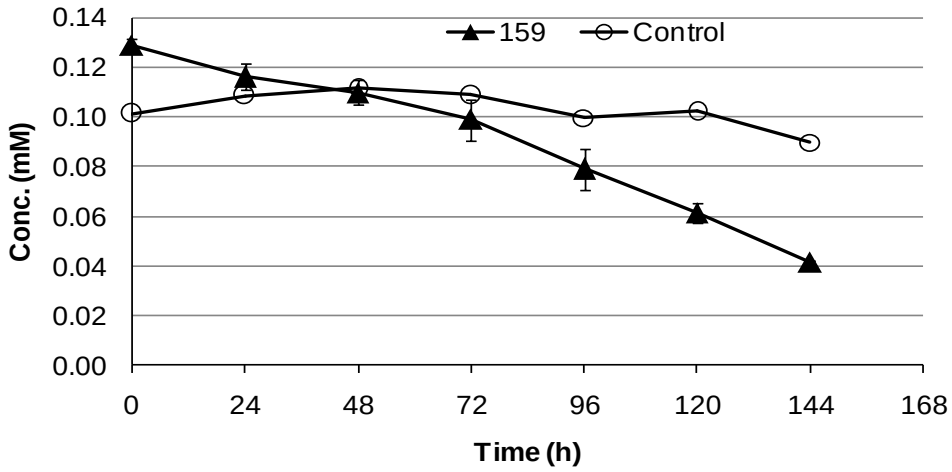


Figure 2.19 Progress curve of desulfoglucobrassicin (**159**) transformation in cultures of *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

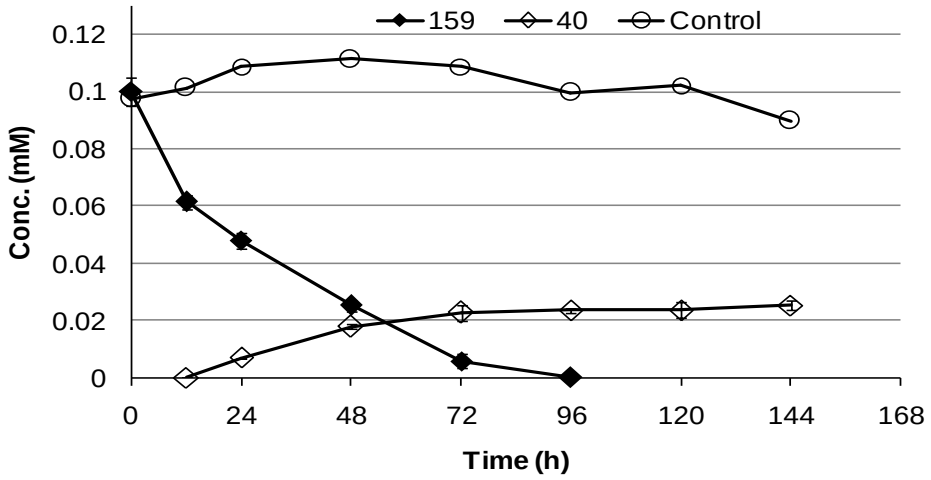


Figure 2.20 Progress curve of desulfoglucobrassicin (**159**) transformation in cultures of *Rhizoctonia solani* and formation of indolyl-3-acetonitrile (**40**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

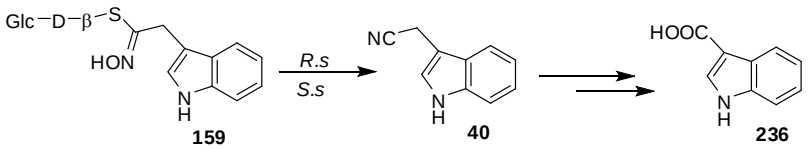


Figure 2.21 Transformation of desulfoglucobrassicin (**159**) by *R. solani* and *S. sclerotiorum*.

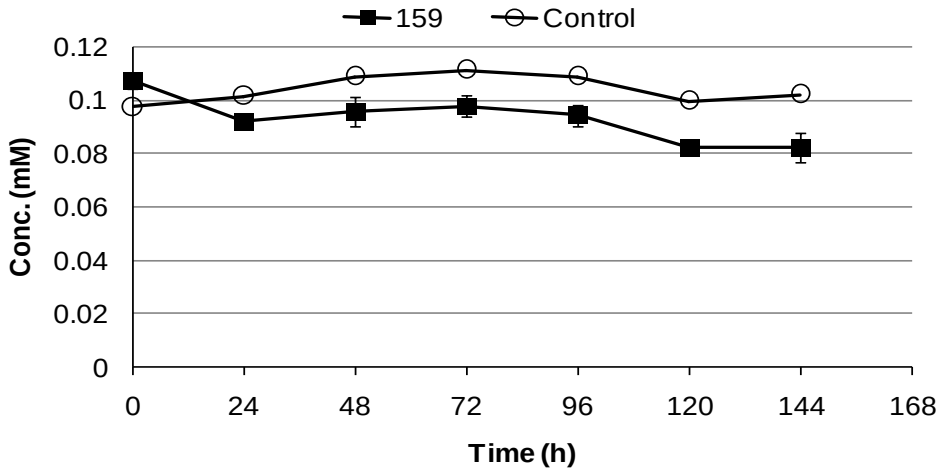


Figure 2.22 Progress curve of desulfoglucobrassicin (**159**) in cultures of *Alternaria brassicicola*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

1-Methoxydesulfoglucobrassicin (233)

1-Methoxydesulfoglucobrassicin (**233**) was also transformed by *R. solani* and S. *sclerotiorum* to 1-methoxyindoly-3-acetonitrile (**102**) which finally transformed to 1-methoxyindolyl-3-carboxylic acid (**237**) (Figure 2.25). The rate of transformation of **233** to **102** by *R. solani* ($t_{1/2}$ = 84 h) (Figure 2.23) is similar to that of *S. sclerotiorum* ($t_{1/2}$ = 72 h) (Figure 2.24), and again *A. brassicicola* did not metabolize **233** (Figure

2.26). Identity of metabolites was confirmed by comparing their HPLC retention time and UV spectra with that of synthetic samples (Pedras and Hossain, 2011).

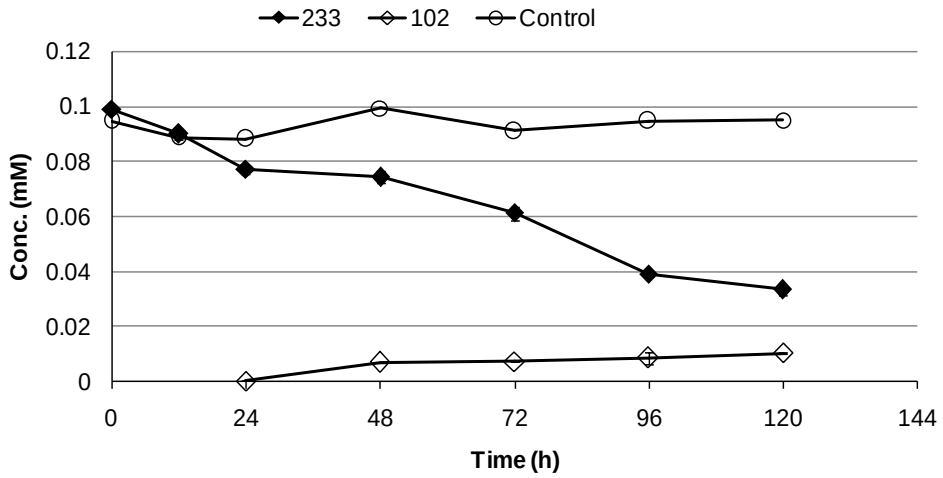


Figure 2.23 Progress curve of 1-methoxydesulfoglucobrassicin (**233**) transformation in cultures of *Rhizoctonia solani* and formation of 1-methoxyindolyl-3-acetonirile (**102**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

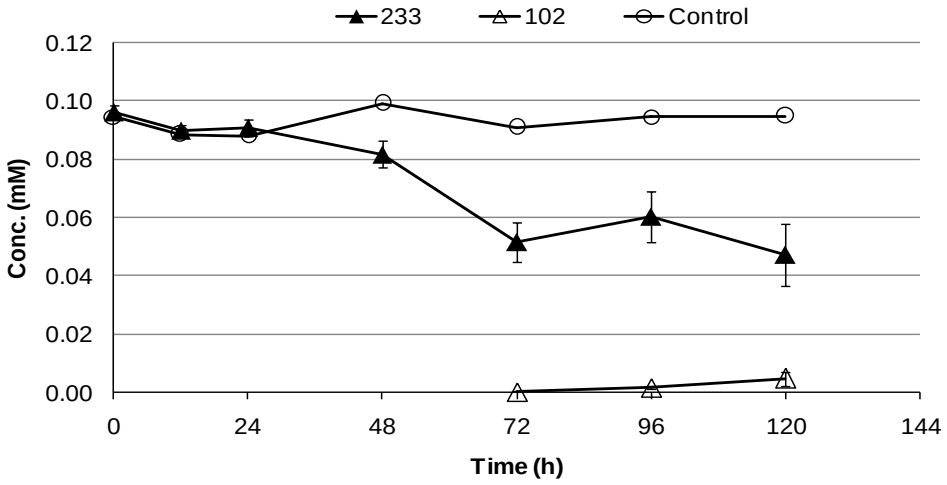


Figure 2.24 Progress curve of 1-methoxydesulfoglucobrassicin (**233**) transformation in cultures of *Sclerotinia sclerotiorum* and formation of 1-methoxyindolyl-3-acetonitrile (**102**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

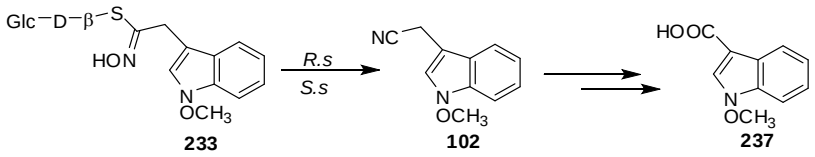


Figure 2.25 Transformation of 1-methoxydesulfoglucobrassicin (**233**) by *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.

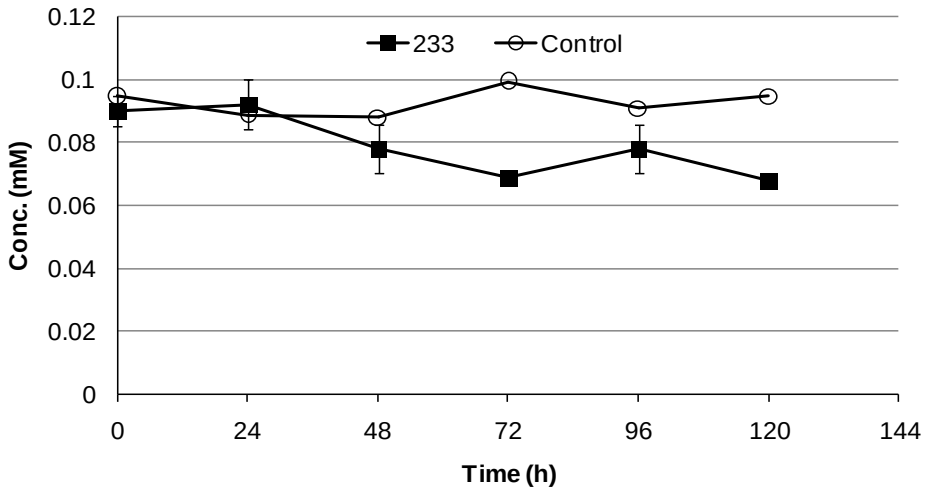


Figure 2.26 Progress curve of 1-methoxydesulfoglucobrassicin (**233**) transformation in cultures of *Alternaria brassicicola*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

Phenyldesulfoglucosinolate (228)

HPLC analysis of concentrated culture samples indicated a complete transformation of phenyldesulfoglucosinolate (**228**) by *A. brassicicola* in 5 days to benzonitrile (**235**) (Figure 2.28) but no transformation was observed by *R. solani* and *S. sclerotiorum* (Figure 2.27). Formation of benzonitrile (**235**) was confirmed by comparing HPLC retention time and UV spectra with that of commercially available sample.

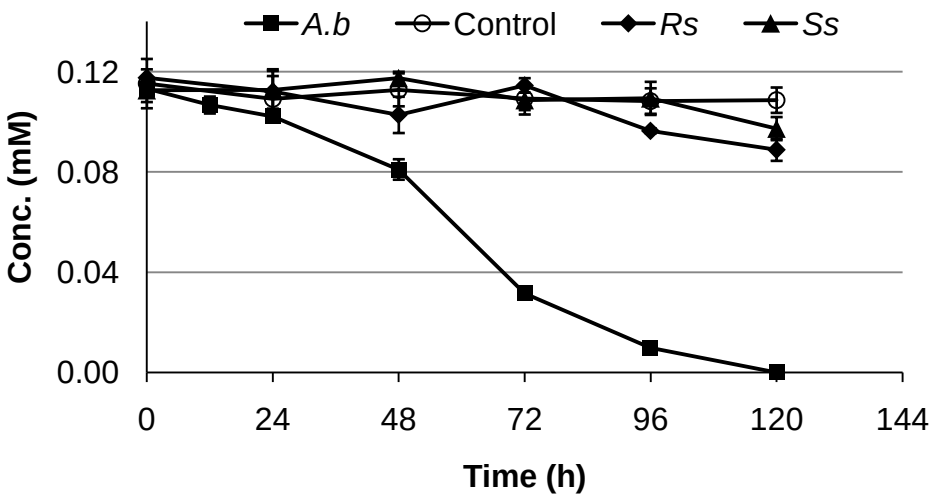


Figure 2.27 Progrss curves of phenyldesulfoglucosinolate (**228**) transformation in cultures of *Alternaria brassicicola, Rhizoctoina solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

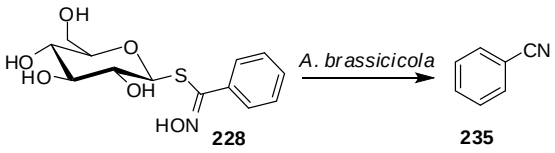


Figure 2.28 Transformation of phenyldesulfoglucosinolate (**228**) by *Alternaria brassicicola.*

Benzyldesulfoglucosinolate (202)

HPLC analysis of concentrated culture samples indicated no transformation of benzyldesulfoglucosinolate (**202**) by *A. brassicicola, R. solani,* and *S. sclerotiorum* (Figure 2.29). The compound **202** was found to be stable in fungal cultures and in H_2O over 5 days.

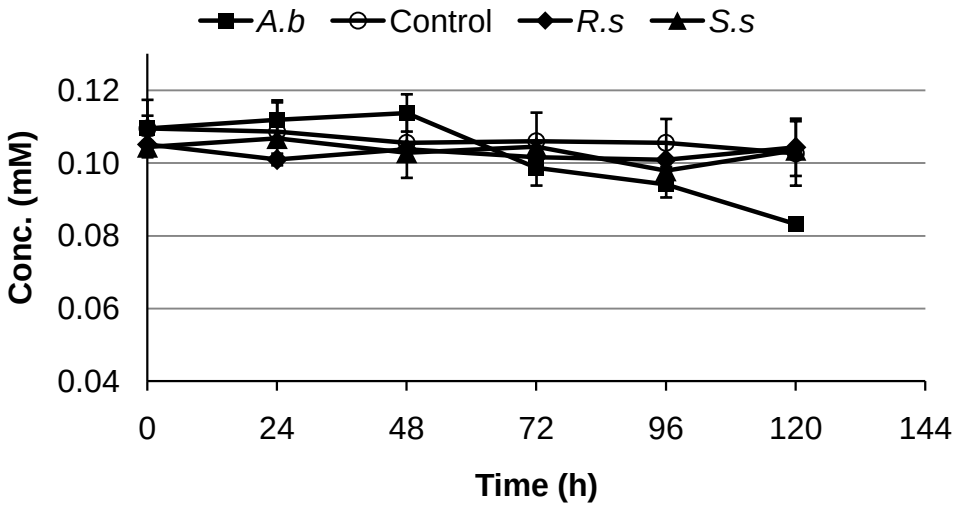


Figure 2.29 Progress curves of benzyldesulfoglucosinolate (**202**) in cultures of *Alternaria brassicicola, Rhizoctonia solani* and *Sclerotinia sclerotiorum* and in H_2O. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

2.2.6. Metabolism of nitriles (**40**, **102**, **103**, **185**, and **235**)

Metabolism of indolyl-3-acetonitrile (**40**), 4-methoxyindolyl-3-acetonitrile (**103**), 1-methoxyindolyl-3-acetonitrile (**102**) (Pedras and Hossain, 2011), phenyl acetonitrile (**185**), and benzonitrile (**235**) by *A. brassicicola, R. solani,* and *S. sclerotiorum* was studied. Nitriles were dissolved in CH_3CN and administered to the cultures of *A. brassicicola*, *R. solani,* and *S. sclerotiorum* separately. Samples were collected at different time intervals up to 5 days, lyophilized and dissolved in CH_3OH:H_2O (1:1) to analyze by HPLC.

Indolyl-3-acetonitrile (40)

Indolyl-3-acetonitrile (**40**) was metabolized by *A. brassicicola* to indole-3-acetic acid (**210**) and finally to indole-3-carboxylic acid (**48**) similar to *R. solani* and *S. sclerotiorum* as reported earlier (Figure 2.31) (Pedras and Montaut, 2003), but the rate of transformation by *A. brassicicola* was faster (100% transformation in 12h) compared to *R. solani* (75% transformation in 5 days) and *S. sclerotiorum* (100 % transformation in 24 h) (Figure 2.30). The transformation of indolyl-3-acetic acid (**210**) to indole-3-carboxylic acid (**48**) was very slow; it remained in the culture in substantial amount even after 96 h. The first step of the biotransformation was the main detoxification step as the antifungal activity of indolyl-3-acetic acid (**210**) was much lower than indolyl-3-acetonitrile (**40**) (Pedras and Hossain, 2011).

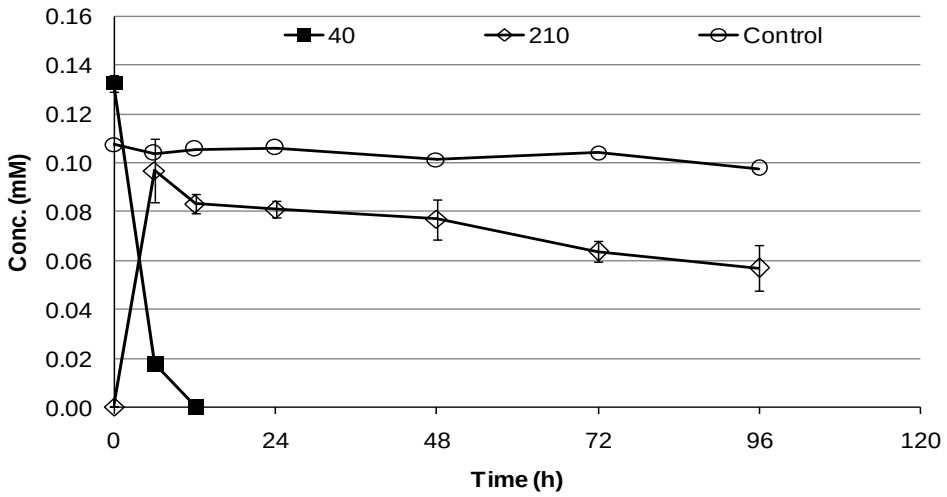


Figure 2.30 Progress curve of indolyl-3-acetonitrile (**40**) tansformation in cultures of *Alternaria brassicicola* and formation of indolyl-3-acetic acid (**210**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

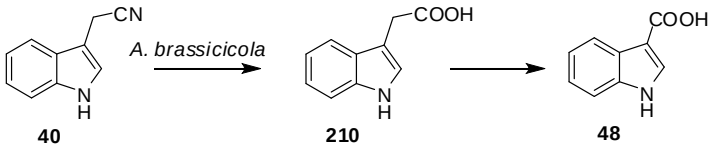


Figure 2.31 Transformation of indolyl-3-acetonitrile (**40**) by *Alternaria brassicicola.*

4-Methoxyindolyl-3-acetonitrile (103)

4-Methoxyindolyl-3-acetonitrile (**103**) was metabolized by *A. brassicicola*, *R. solani* and *S. sclerotiorum* to 4-methoxyindolyl-3-acetic acid (**238**) and finally to 4-methoxyindolyl-3-carboxylic acid (**239**) (Figure 2.33). Metabolism by *R. solani* was faster (50% in ca. 18 h) than by *A. brassicicola* and *S. sclerotiorum* (50% in ca. 48 h) (Figure 2.32). Formation of 4-methoxyindoyl-3-acetic acid (**238**) and 4-methoxyindoyl-3-carboxylic acid (**239**) was confirmed by LC, LC-MS, and UV spectral data with that of the synthetic sample (Pedras and Hossain, 2011).

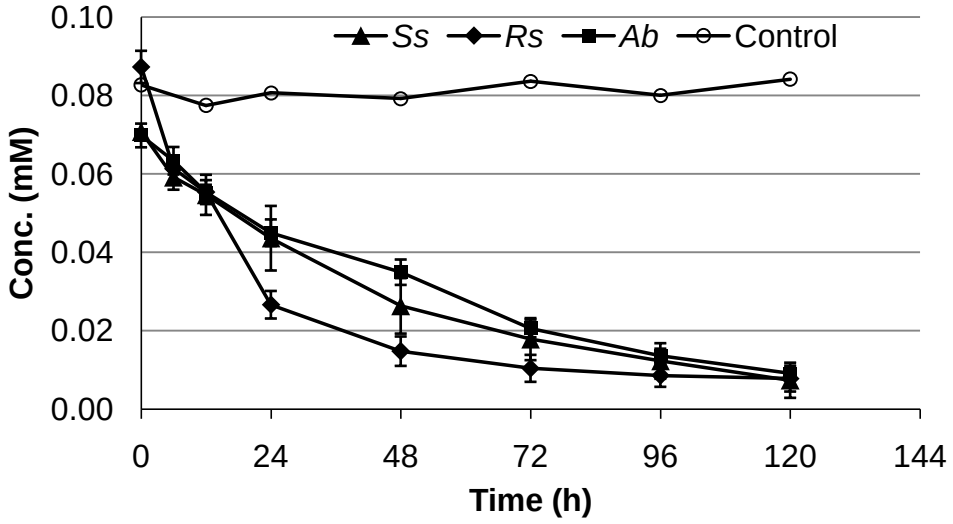


Figure 2.32 Progress curves of 4-methoxyindolyl-3-acetonitrile (**103**) transformation in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

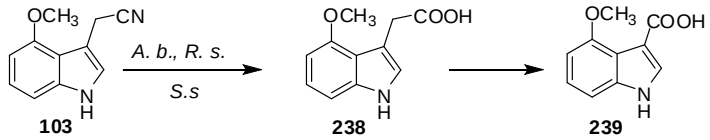


Figure 2.33 Transformation of 4-methoxyindolyl-3-acetonitrile (**103**) by *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.

1-Methoxyindolyl-3-acetonitrile (102)

1-Methoxyindolyl-3-acetonitrile (**102**) was metabolized by *A. brassicicola*, *R. solani* and *S. sclerotiorum* to 1-methoxyindolyl-3-acetic acid (**240**) and finally to 1-methoxyindolyl-3-carboxylic acid (**241**) (Figure 2.35). The rate on transformation of 1-methoxyindolyl-3-acetonitrile (**102**) was different in all cases. Transformation was the fastest in cultures of *A. brassicicola* ($t_{1/2}$ = 24 h) and slowest in the case of *S. sclerotiorum* ($t_{1/2}$ = 68 h) and in the case of *R. solani* $t_{1/2}$ was 48 h (Figure 2.34).

Conversion of 1-methoxyindolyl-3-acetic acid (**240**) to 1-methoxyindolyl-3-carboxylic acid (**241**) was very fast. Formation of 1-methoxyindoyl-3-acetic acid (**240**) and 1-methoxyindoyl-3-caroboxylic acid (**241**) was confirmed by LC, LC-MS and UV spectra with that of the synthetic sample (Pedras and Hossain, 2011).

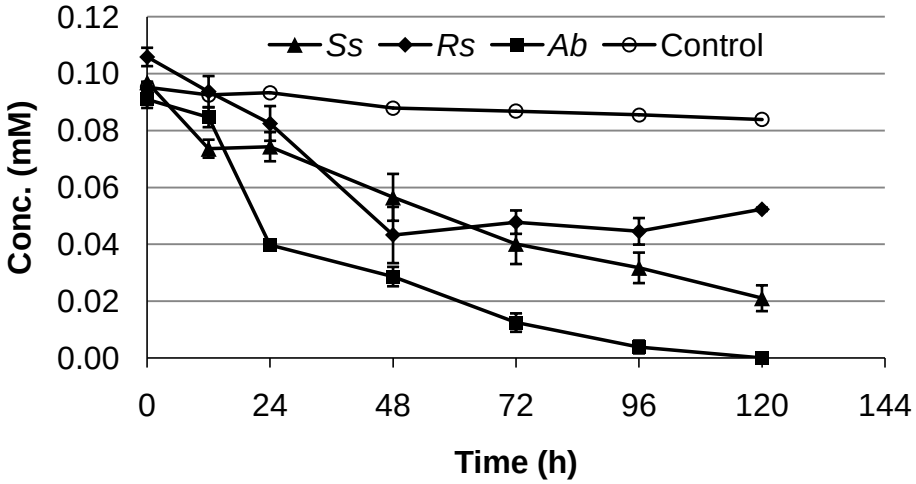


Figure 2.34 Progress curve of 1-methoxyindolyl-3-acetonitrile (**102**) transformation in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

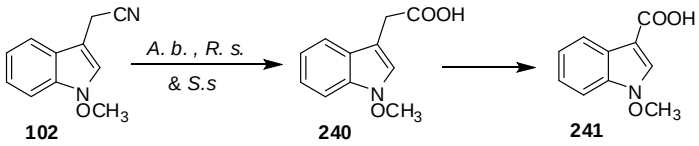


Figure 2.35 Transformation of 1-methoxyindolyl-3-acetonitrile (**102**) by *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.

Phenyl acetonitrile (185)

HPLC analysis of concentrated culture samples indicated transformation of phenyl acetonitrile (**185**) by *A. brassicicola* and *S. sclerotiorum* to phenyl acetic acid (**199**) and finally to benzoic acid (**242**) (Figure 2.36) (100 % transformation in 12 h) but no transformation was observed by *R. solani* (Figure 2.37).

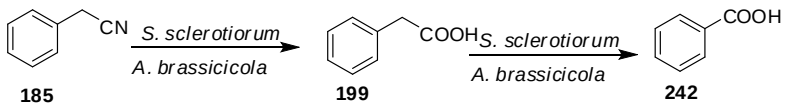


Figure 2.36 Metabolism of phenyl acetonitrile (**185**) by *Alternaria brassicicola* and *Sclerotinia sclerotiorum.*

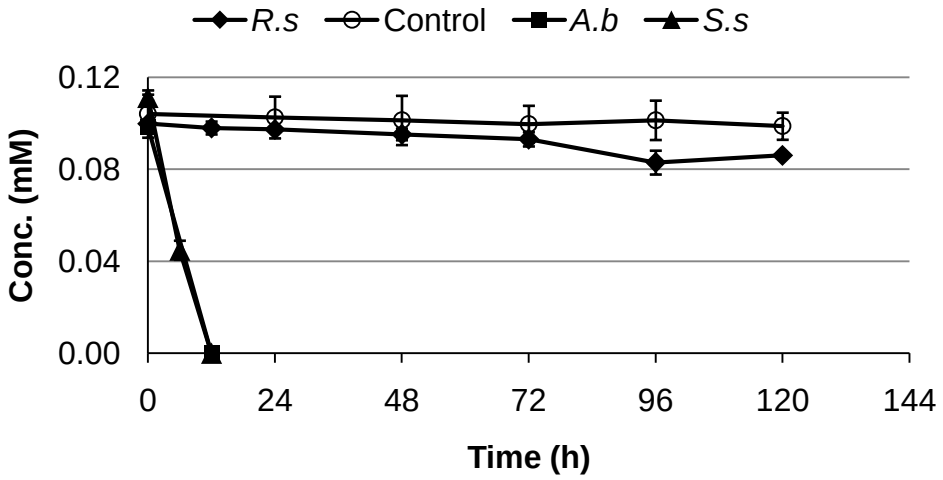


Figure 2.37 Progress curves for phenyl acetonitrile (**185**) transformation in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

Benzonitrile (235)

Benzonitrile (**235**) was metabolized to benzoic acid (**242**) by *S. sclerotiorum* (Figure 2.38) (100% in 96 h) only but not by *A. brassicicola* or *R. solani* (Figure 2.39).

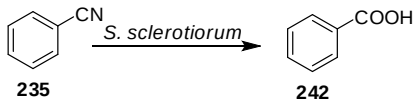


Figure 2.38 Transformation of benzonitrile (**235**) by *Sclerotinia sclerotiorum*.

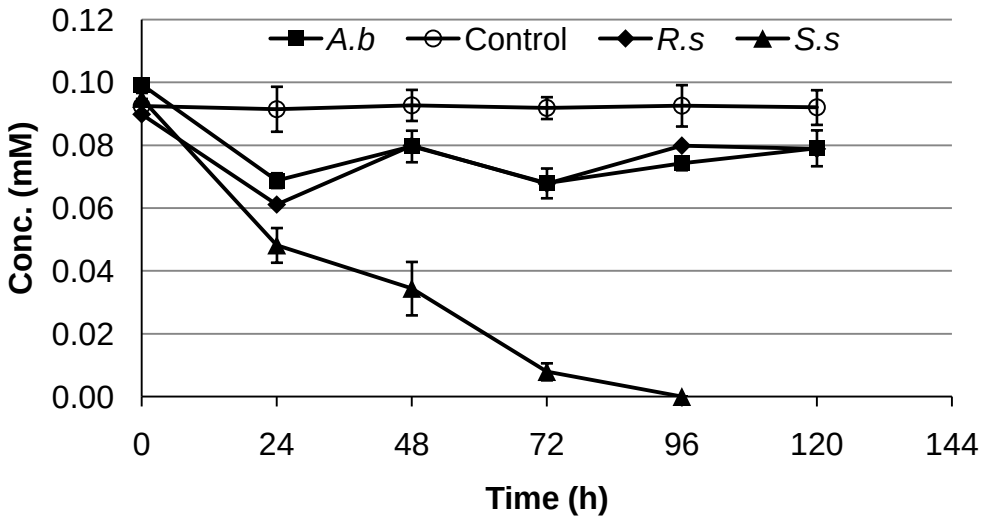


Figure 2.39 Progress curve of benzonitrile (**235**) transformation in cultures of *Alternaria brassicicola, Rhizoctonia solani* and *Sclerotinia sclerotiorum.* Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

2.2.7. Metabolism of indolyl-3-methanol (110), ascorbigen (107), diindolyl-3-methane (113), and isothiocyanates (170 and 234)

To study the metabolism of indolyl-3-methanol (**110**), ascorbigen (**107**), diindolyl-3-methane (**113**), and isothiocyanates (**170** and **234**) stability of these compounds were tested in H_2O. All these compounds were found as sufficiently stable in H_2O (Pedras and Hossain, 2011).

Indolyl-3-methanol (110)

Inldolyl-3-methanol (**110**) was dissolved in CH_3CN and added to the cultures of *A. brassicicola*, *R. solani,* and *S. sclerotiorum* separately to get the final concentration 0.10 mM. Samples were withdrawn immediately and at different intervals, immediately frozen, lyophilized, and dissolved in CH_3CN for analyses by HPLC. Indolyl-3-methanol (**110**) was metabolized very quickly by both *A. brassicicola* and *R. solani* in (100% metabolism in ca. 12 h) (Figure 2.40 and 2.41) to indole-3-carboxaldehyde (**47**) and then to indole-3-carboxylic acid (**48**) (Figure 2.40). The identity of both the metabolites was confirmed against authentic commercially available compounds. Metabolism by *R. solani* (80% metabolism by 6h) was faster than by *A. brassicicola* (50% metabolism by 6h). Transformation of **47** to **48** was also confirmed by feeding of **47** separately to both the fungi. The rate of transformation of **47** was also faster in case of *R. solani* than *A. brassicicola* (Pedras and Hossain, 2011).

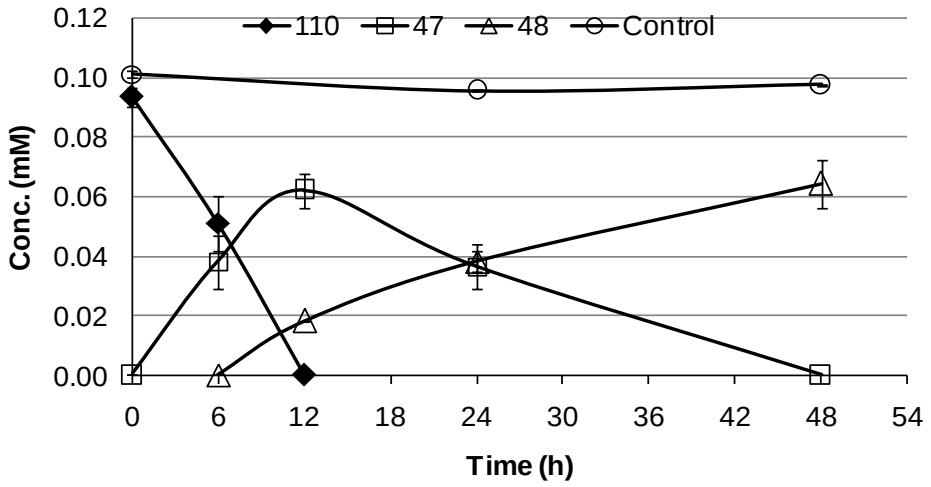


Figure 2.40 Progress curve of indolyl-3-methanol (**110**) transformation by *Alternaria brassicicola* and formation of indole-3-carboxaldehyde (**47**) and indole-3-carboxylic acid (**48**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

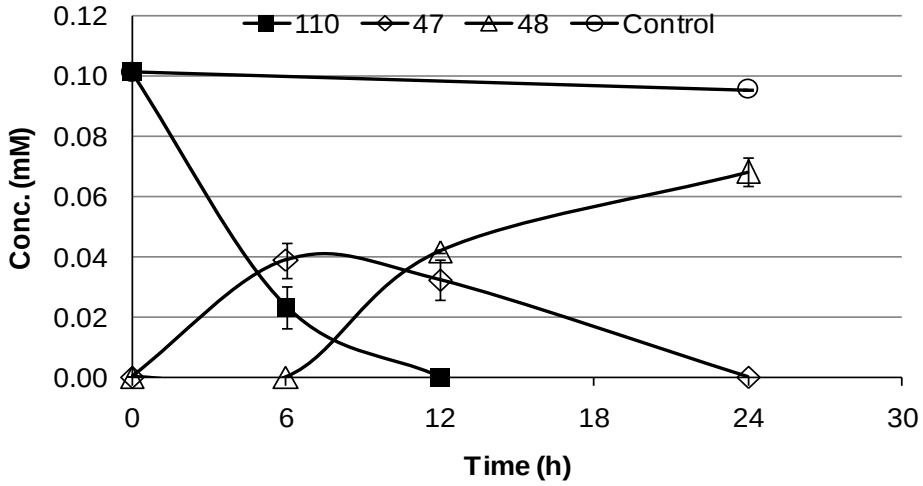


Figure 2.41 Progress curve of indolyl-3-methanol (**110**) transformation by *Rhizoctonia solani* and formation of indole-3-carboxaldehye (**47**) and indole-3-carboxylic acid (**48**). Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

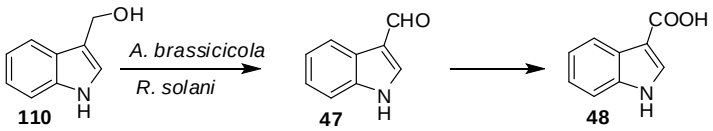


Figure 2.42 Transformation of indolyl-3-methanol (**110**) by *Alternaria brassicicola* and *Rhizoctonia solani*.

Transformation of indolyl-3-methanol (**110**) could not be studied in *S. sclerotiorum* culture since **110** decomposed quickly in cultures of *S. sclerotiorum*; several compounds were detected in the concentrated sample analyzed by HPLC. Isolation and characterization by ^{1}H NMR spectroscopic data and EI-HRMS showed that the main decomposition products are diindolyl-3-methane (**113**) and **243** that resulted from dimerization and trimerization of **110**, respectively, as shown in Figure 2.43. This decomposition occurred likely via formation indolyl-3-methylene cation (**104**) from indolyl-3-methanol (**110**). This decomposition was eventually found to be due to the acidic nature of the culture solution; it was found that in water mycelia of *S. sclerotiorum* excreted sufficient amounts of acids to decrease the pH of the culture solution to 3.8. The low pH of medium was probably due to the production of oxalic acid by *S. sclerotiorum*, which has been reported under different conditions (Bolton et al., 2006). Oligomerization of indolyl-3-methanol (**110**) in acidic conditions was reported earlier by Grose and Bjeldanes (1992). To avoid the excess acid formed from fungus, the fungal mycelia were washed several times with sterile water but the pH of the fungal culture did not change much. Keeping the fungal culture overnight in water and filtering out the broth did not help either. In the case of *A. brassicicola* and *R. solani*, the pH of the culture medium remained around 6.5 (Pedras and Hossain, 2011).

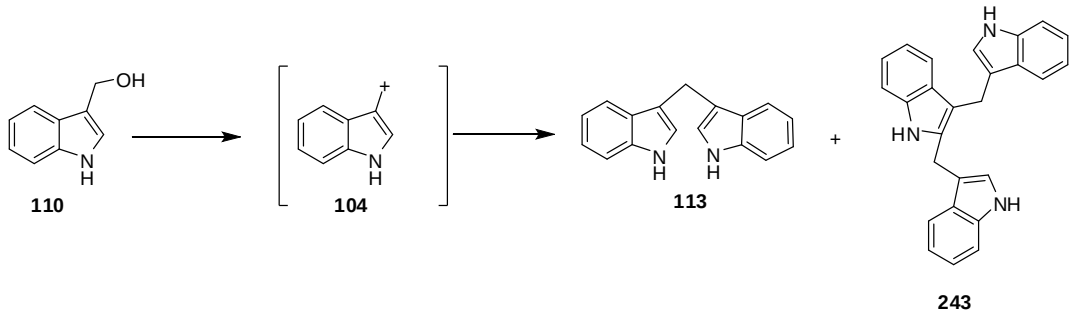


Figure 2.43 Transformation of indolyl-3-methanol (**110**) in cultures of *Sclerotinia sclerotiorum*.

Metabolism of ascorbigen (107), diindolyl-3-methane (113) and isothiocyanates (170 and 234)

Incubation of ascorbigen (**107**) and diindolyl-3-methane (**113**) with each pathogen and analyses of concentrated cultures of HPLC indicated no additional compounds present in the culture. Ascorbigen (**107**) was not very stable in water and showed ca. 40% decomposition after 96 h (Figure 2.44) (Pedras and Hossain, 2011).

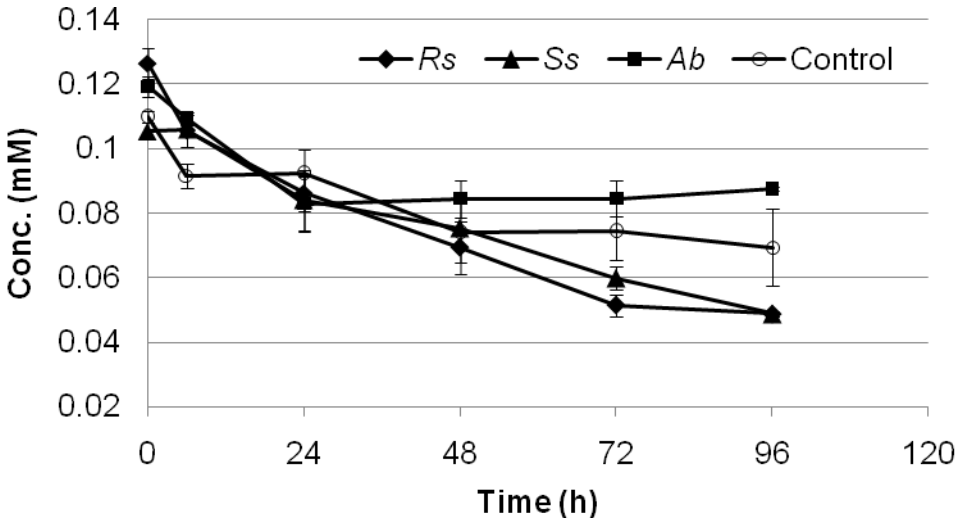


Figure 2.44 Progress curves of ascorbigen (**107**) in H_2O and in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

The major problem of working with diindolyl-3-methane (**113**) was its very low solubility in water. Using excess amount of organic solvent (CH_3CN) only improved the solubility merginally. About 50% of diindolyl-3-methane (**113**) was precipitated from the media, which was then adsorbed into the fungal mycelia. The recovery of **113** from cultures was ca. 90% after 5 days, 50% was recovered from the broth and 40% from the fungal mycelia. Benzyl isothiocyanate (**170**) and phenyl isothiocyanate (**234**) were too volatile to study their metabolism in culture media. Both the compounds (**170** and **234**) were dissolved in CH_3CN and were added to H_2O to study the stability of the compounds in H_2O. When the samples were collected, freeze-dried, and analyzed by HPLC, no compounds were detected. The concentration of benzyl isothiocyanate (**170**) decreased to 70% in 72 h from aquous solution in an Erlenmeyer flask whereas, the concentration of phenyl isothiocyanate (**234**) decreased to 90% in 6 h under similar conditions. The evaporation rate was much slower when the compounds were kept in a

closed HPLC vials. Benzyl isothiocyanate was about 85% after 72 h (Figure 2.45) and phenyl isothiocyanate was about 70% after 72 h (Figure 2.46).

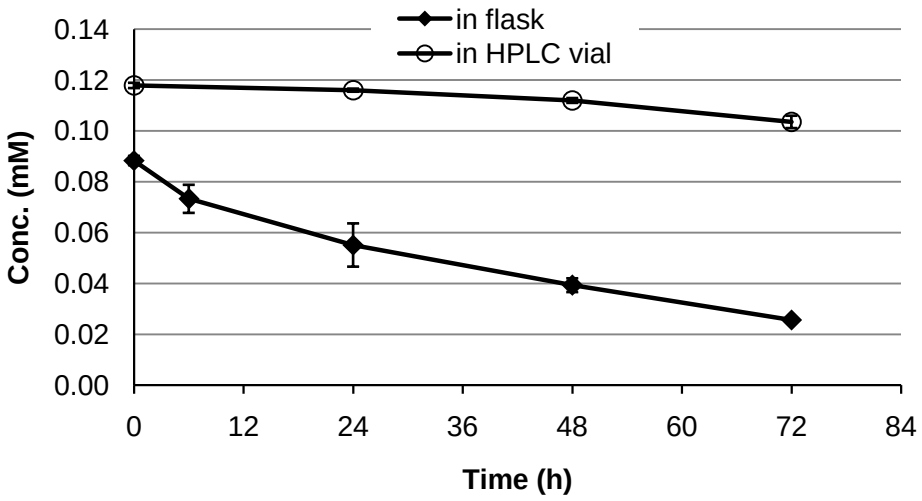


Figure 2.45 Benzyl isothiocyanate (**170**) in H_2O. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

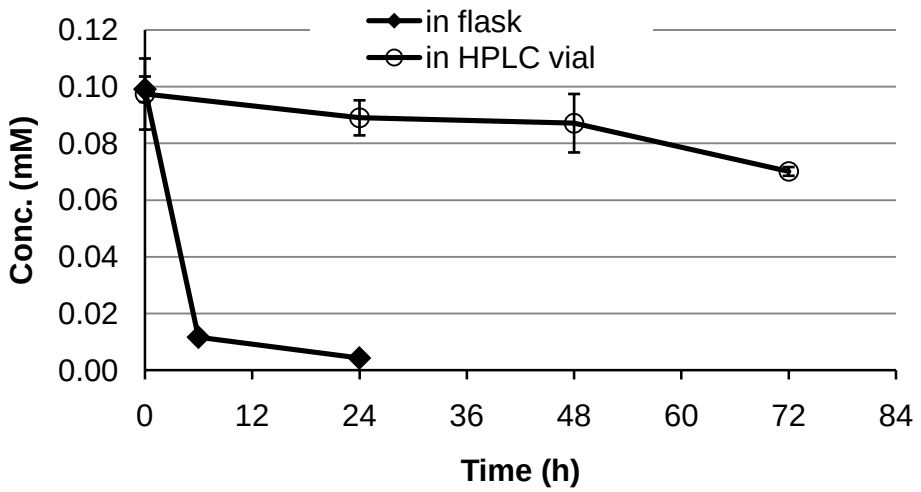


Figure 2.46 Phenyl isothiocyanate (**234**) in H_2O. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

Benzyl isothiocyanate (**170**) was then added to the fungal cultures of *A. brassicicola* at a concentration of 0.10 mM. Benzyl isothiocyanate (**170**) completely was not detcted in fungal cultures of *A. brassicicola* after 24h of incubation, but no metabolite was detected. On the other hand, **170** was found to be stable in fungal cultures of *R. solani* and *S. sclerotiorum* as the rate of disappearance was similar to that of control (Figure 2.47). To find the fate of benzyl isothiocyanate (**170**) in cultures of *A. brassicicola* the mycelia was extracted with methanol and analyzed by HPLC, but no extra peak was observed other than those in the control experiment. To find the probable metabolites both broth and mycelial extract were tested for the presence of benzyl amine and benzyl alcohol but none of the compounds were found in either broth or in mycelial extracts (limit of detection for benzyl alcohol and benzyl amine is 1 nmol).

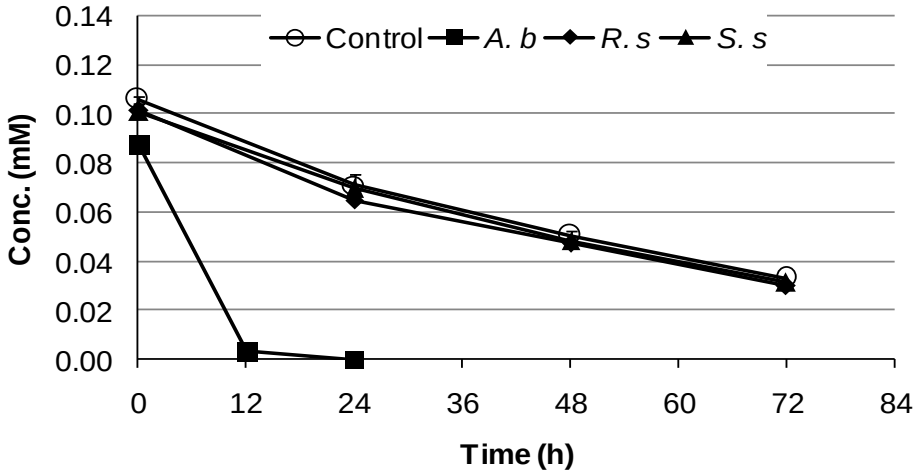
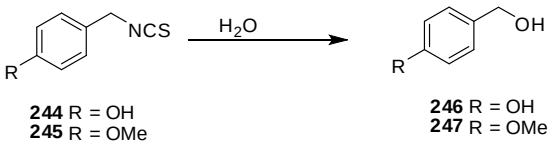


Figure 2.47 Progress curves of benzyl isothiocyanate (**170**) transformation in H_2O and in cultures of *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Concentrations were determined using calibration curves; each point is the average of at least three independent experiments ± standard deviations.

Two other isothiocyanates, 4-hydroxybenzyl isothiocyanate (**244**) and 4-methoxybenzyl isothiocyanate (**245**) were synthesized (Pedras and Smith, 1997), but unfortunately, both compounds were found to be unstable in water and were transformed into the corresponding alcohol. Transformation of 4-methoxybenzyl isothiocyanate (**245**) into 4-methoxybenzyl alcohol (**247**) was slower compared to transformation 4-hydroxybenzyl isothiocyanate (**244**) into 4-hydroxybenzyl alcohol (**246**). 4-Hydroxybenzyl isothiocyanate (**244**) was almost immediately transformed into the alcohol **246** whereas 4-methoxybenzyl isothiocyanate (**245**) was transformed ca. 75% into the corresponding alcohol (**247**) in 72 h (Figure 2.48).



244 R = OH
245 R = OMe

246 R = OH
247 R = OMe

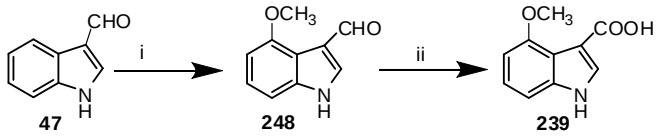
Figure 2.48 Transformation of 4-hydroxybenzyl isothiocyanate (**244**) and 4-methoxybenzyl isothiocyanate (**245**) in H_2O.

2.2.8. Synthesis and antifungal activities of metabolites

Synthesis of 4-methoxyindole-3-carboxylic acid (**239**) was achieved successfully for the first time. 1-Methoxyindole-3-carboxylic acid (**242**) was synthesized following a published procedure (Somei et al., 2001). Commercially available compounds were used as such after confirming their purity by HPLC. Antifungal activities of metabolites of glucosinolates and derivatives were determined against *A. brassicicola*, *R. solani*, and *S. sclerotiorum* at different concentrations using DMSO as the solvent.

Synthesis of 4-methoxyindole-3-carboxylic acid (239)

Synthesis of 4-methoxyindole-3-carboxylic (**239**) was achieved by following a published procedure with slight modification as per scheme 2.7. At first, the synthesis of 4-methoxyindole-3-carboxaldehyde (**248**) was carried out by methoxylation of indole-3-carboxaldehyde (**47**) in 75% yield (Somei et al., 1984). 4-Methoxyindole-3-carboxaldehyde (**248**) was then oxidized by $NaClO_2$ by following the procedure for the synthesis of 1-methoxyindole-3-carobxylic acid (**239**) (Somei et al. 2001).



Scheme 2.7 Synthesis of 4-methoxyindole-3-carboxylic acid (**239**). Reagents and conditions: (i) $Tl(CF_3COO)_3$-TFA, I_2, CuI, DMF, r.t., NaOMe, reflux, 75%; (ii) $NaClO_2$, NaH_2PO_4. $2H_2O$, 2-methyl-2-butene, 91% (Somei et al., 1984; 2001).

Antifungal activity of metabolites

Antifungal activities of metabolites of glucosinolate derivatives (**210**, **47**, **48**, **237**, **239**, **199**, and **242**) were determined against *A. brassicicola*, *R. solani*, and *S. sclerotiorum* at different concentrations using DMSO as the solvent and the results are summarized in Table 2.5.

Table 2.5 Antifungal activity of metabolites of glucosinolate derivatives (**210**, **47**, **48**, **237**, **239**, **199**, and **242**) against the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light.

Compound name	Conc. (mM)	% Inhibition (± S. E.)*		
		A. brassicicola	*R. solani*	*S. sclerotiorum*
Indolyl-3-acetic acid (**210**)	0.50	28 (2)$^{a, x}$	19(1)$^{a, y}$	18 (1)$^{a, y}$
	0.20	13(3)	12(0.3)	6 (0)
	0.10	NI	7 (0)	NI
Indole-3-carboxylic acid (**48**)	0.50	22 (0)$^{b, x}$	9 (0.3)$^{b, y}$	34 (2)$^{b, z}$
	0.20	11 (0)	5 (0.3)	9 (0)
	0.10	6 (0)	NI	4 (0)
1-Methoxyindole-3-carboxylic acid(**237**)	0.50	48 (1)$^{c, x}$	14 (0)$^{c, y}$	67 (0)$^{c, z}$
	0.20	24 (0)	7 (0)	43 (1)
	0.10	12 (0)	3 (0.3)	27 (0)
4-Methoxyindole-3-carboxylic acid (**239**)	0.50	18 (0)$^{b, x}$	14 (0)$^{c, y}$	11 (0.3)$^{d, y}$
	0.20	11 (1)	6 (0.3)	3 (0)
	0.10	7 (1)	NI	NI
Indole-3-carboxaldehyde (**47**)	0.50	29 (1)$^{a, x}$	32 (1)$^{d, x}$	16 (0.3)$^{a, y}$
	0.20	21 (1)	17 (1)	6 (0)
	0.10	6 (1)	7 (1)	NI
Phenyl acetic acid (**199**)	0.50	14 (1)$^{d, x}$	18 (0.6)$^{a, y}$	8 (0.9)$^{d, z}$
	0.20	9 (1)	10 (1)	0
	0.10	0	0	0
Benzoic acid (**242**)	0.50	17 (0.4)$^{d, x}$	0$^{e, y}$	11 (0.6)$^{d, z}$
	0.20	0	0	5 (0.7)
	0.10	0	0	0

*Percentage of growth inhibition calculated using the formula: % inhibition = 100 - [(growth on amended medium/growth in control medium) - 100]. Data are the mean ± S. E.; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted a set at 0.05; n = 3. Different letters in the same column (a–e) indicate significant differences ($P < 0.05$); different letters in the same row (x–z) indicate significant differences ($P < 0.05$).

2.2.9. Myrosinase activity

Myrosinase activity in cruciferous fungal pathogens has not been reported earlier. Several *Aspergillus* species have been reported to have myrosinase activity and being able to transform several glucosinolates (James and Rossiter, 1991). To determine myrosinase activity in *A. brassicicola*, and also screen for the activity in *R. solani* and *S. sclerotiorum* cultures were grown in MM for couple of days. Cell free extracts from mycelia of each fungus was prepared as described in the experimental. Myrosinase activity of cell-free extracts of mycelia of *A. brassicicola* grown in minimal media for 3 days was determined using glucobrassicin as a substrate. The major products of the enzymatic reactions were indolyl-3-methanol (**110**) and diindolyl-3-methane (**113**). The amounts of products were determined by HPLC. Crude extract of *A. brassicicola* showed a low but consistent myrosinase activity against glucobrassicin (**86**). 1-methoxyglucobrassicin (**87**) was tried as an inducer but no significant difference in activity was observed. Ascorbate has been reported to increase myrosinase activity (Shikita et al., 1999) but was not used for this particular experiment to avoid the interaction with indolyl-3-methanol (**110**) and form ascorbigen (**107**). The substrate specificity of the crude extracts was tested against sinigrin (**131**), phenylglucosinolate (**65**), benzylglucosinolate (**66**), glucobrassicin (**86**), and 1-methoxyglucobrassicin (**87**) using 0.3 mM sodium ascorbate in the reaction mixture. Myrosinase activity was determined by calculating the concentration of glucose formed due to myrosinase activity on glucosinolates. Total content of glucose was measured using a glucose assay kit (Sigma). Glucose was phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) was then oxidized to 6-phospho-gluconate in the presence of nicotinamide

adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration. The procedure is described in experimental and the results are summarized in table 2.6. Myrosinase activity of *R. solani* and *S. sclerotiorum* was also tested similarly in the crude exact of mycelia but none of the pathogens showed myrosinase activity against any of the glucosinolates tested.

Table 2.6 Myrosinase activity of crude cell-free extract of *Alternaria brassicicola*

Compound	Specific activity (nmols. $mg^{-1}min^{-1}$)			
	Expt. 1	Expt. 2	Expt. 3	Ave.(± S. D.)
Sinigrin (**131**)	2.19	2.35	2.42	2.32 (0.11)
Phenylglucosinolate (**65**)	1.98	2.61	1.82	2.14 (0.42)
Benzylglucosinolate (**66**)	2.19	2.61	2.06	2.29 (0.28)
Glucobrassicin (**86**)	1.03	1.57	0.96	1.19 (0.33)
1-Methoxyglucobrassicin (**87**)	1.03	1.70	1.15	1.29 (0.35)

Myrosinase activity of a commercially available plant thioglucosidase (thioglucosidase from *Sinapis alba* (white mustard) seed, ≥100 units/g solid; one unit produce 1.0 micromole of glucose per minute from sinigrin at pH 6.0 at 25 °C) was also determined in parallel to compare the activities between fungal myrosinase and plant myrosinase.

The cell-free extracts of mycelia of *A. brassicicola* displayed higher myrosinase activity for sinigrin (**131**), phenyl and benzyl glucosinolates (**65** and **66**) but lower

activities with glucobrassicin (**86**) and 1-methoxyglucobrassicin (**87**) on the other hand, commercial myrosinase from *S. alba* displayed similar myrosinase activity for sinigrin (**131**), benzylglucosinolate (**66**), glucobrassicin (**86**) and 1-methoxyglucobrassicin (**87**) but very little activity with phenylglucosinolate (**65**).

Table 2.7 Myrosinase activity of commercial myrosinase (thioglucosidase from *Sinapis alba* seed)

Compound	Specific activity (nmols. $mg^{-1}min^{-1}$)			
	Expt. 1	Expt. 2	Expt. 3	Ave.(± S. D.)
Sinigrin (**131**)	54.0	55.0	53.4	54.1 (0.8)
Phenyl glucosinolate (**65**)	3.7	7.9	-	5.8 (3.0)
Benzylglucosinolate(**66**)	37.0	51.5	47.2	45.2 (7.4)
Glucobrassicin (**86**)	34.6	40.5	40.5	38.5 (3.4)
1-Methoxyglucobrassicin (**87**)	43.2	50.2	-	46.7 (5.0)

2.2.10. Discussion

Neither glucosinolates nor their desulfo-derivatives displayed any detectable antifungal activity against any of the fungus tested. Diindolyl-3-methane (**113**) was most active of all compounds, inhibiting growth of the three fungal species completely at 0.50 mM, ascorbigen (**107**) displayed the lowest activity. Benzyl isothiocyanate (**170**) showed very high inhibitory effect against *R. solani* and *S. sclerotiorum* (100% at 0.50 mM) but low inhibitory effect against *A. brassicicola* (12% at 0.50 mM). Phenyl isothiocyanate (**234**) showed higher inhibition against *S. sclerotiorum* (63% at 0.50

mM) than *R. solani* (36% at 0.50 mM) and no inhibition against *A. brassicicola*. Among the nitriles, 1-methoxyindolyl-3-acetontrile (**102**) was most active against *R. solani,* 100% inhibition at 0.50 mM but showed lower inhibition against *A. brassicicola* and *S. sclerotiorum* (43% and 54% respectively). 4-Methoxyindolyl-3-acetonitrile (**103**) displayed higher inhibition against *R. solani* and *S. sclerotiorum* than against *A. brassicicola* (70%, 77%, and 59 % respectively at 0.50 mM). Phenyl acetonitrile (**185**) showed higher inhibition than benzonitrile (**235**) against *R. solani* and *S. sclerotiorum* but none of the compounds showed inhibition against *A. brassicicola*. Indolyl-3-methanol (**110**) showed moderate inhibitory activity against *A. brassicicola* and *R. solani* but high inhibition against *S. sclerotiorum* (43%, 44%, and 73% respectively).

Two aryl glucosinolates (**65** and **66**) were metabolized by the host-specific pathogen *A. brassicicola* to the corresponding nitriles (**185** and **235**) and finally to corresponding acids (**242** and **199**) but not by the two non host-specific pathogens *R. solani* and *S. sclerotiorum* whereas, none of the indolylglucosinolates were metabolized by any of the pathogens. Although a slight decrease in concentration of 1-methoxyglucobrassicin (**87**) was detected, this was similar to that observed in control solutions and attributed to its slow decomposition. Metabolism of benzylglucosinolate (**66**) was faster (100% in 24h) than phenylglucosinolate (**65**) (100% in 72 h). To check whether the enzyme responsible for this transformation is secreted outside cell, the fungus was grown in MM and the mycelia was transferred to water after three days. The fungal mycelia were then filtered out after 24 h and glucosinolates (**65** and **66**) were added to the broth but no transformation was observed up to 4 days. This result indicates that the enzyme probably does not secrete outside the cell rather a specific carrier was needed to take the sample inside the fungal cell. Indolyl desulfoglucosinolates (**159** and **233**) were metabolized by two non-host specific fungi

R. solani and *S. sclerotiorum* to the corresponding nitriles (**40** and **102**) and finally to corresponding carboxylic acids (**48** and **241**) but not by the host-specific *A. brassicicola*. A slow metabolism of phenyldesulfoglucosinolate (**228**) was observed by *A. brassicicola* (50% in about 60 h) to benzonitrile (**235**) but no metabolism was observed in case of *R. solani* and *S. sclerotiorum*, whereas benzyldesulfoglucosinolate (**202**) was found to be stable in fungal cultures of *A. brassicicola, R. solani* and *S. sclerotiorum*. This seems to be the first observed transformation of glucosinolates and desulfoglucosinolates by cruciferous pathogenic fungi (Pedras and Hossain, 2011). Previously, a soil isolate of fungus *Aspergillus flavus* was shown to metabolize 2-propenyl and 2-phenylethyl glucosinolates (**131** and **67**) and their desulfo-derivatives to the corresponding nitriles using a sulfatase (Galletti et al., 2008) but no other fungal pathogen has been reported for metabolism of glucosinolates. Metabolism of two aryl glucosinolates (**65** and **66**) by *A. brassicicola* reveals the presence of a myrosinase type enzyme in *A. brassicicola*, which was later confirmed, in crude cell free extract of *A. brassicicola*. Metabolism of desulfo-IGLs suggests that both *R. solani* and *S. sclerotiorum* produce glucosyl hydrolyses that do not have myrosinase activity, and do not produce sulfatases, as none of the glucosinolates were metabolized. However, these transformations of glucosinolates and desulfoglucosinolates were not detoxification processes, since the metabolites were more inhibitory to each fungal species than the corresponding glucosinolates and desulfoglucosinolates.

Indolyl acetonitriles (**40**, **102**, and **103**) were metabolized by each species to the corresponding carboxylic acids similar to transformations occurring *in planta.* Interestingly, the inhibitory activity of nitriles (**40** and **103**) was higher against *R. solani*, than that of acids (**48**, and **239**) (41% vs. 9% at 0.50 mM) and (100% vs. 14% at 0.50 mM), respectively, whereas 1-methoxyindole-3-caroboxylic acid (**237**) was more

inhibitory to *S. sclerotiorum* than the corresponding nitrile (**102**) (54% vs. 67% at 0.50 mM). By contrast, 4-methoxyindolyl-3-acetonitrile (**103**) was more inhibitory to the three species than the corresponding acid (**239**) (59% vs. 18% against *A. brassicicola*, 70% vs. 14% and *R. solani*, and 77% vs. 11% against *S. sclerotiorum* at 0.50 mM). Phenyl acetonitrile (**185**) was metabolized by *A. brassicicola* and *S. sclerotiorum* (100% in 6h) to phenyl acetic acid (**199**) but not by *R. solani*. Benzonitrile (**235**) was metabolized by only *S. sclerotiorum* to benzoic acid (**242**) but not by *A. brassicicola* and *R. solani*. Metabolisms of two aryl nitriles to acids are detoxification reactions as corresponding acids are less inhibitory than the nitriles. Phenyl acetonitrile (**185**) is more inhibitory to *A. brassicicola* and *S. sclerotiorum* than phenyl acetic acid (**199**) and benzonitrile (**235**) is more inhibitory to *S. sclerotiorum* than benzoic acid (Table 2.6 and Table 2.7). Indolyl-3-methanol (**110**) was metabolized to indole-3-carboxaldehyde (**47**) and then to indole-3-carboxylic acid (**48**) by both *A. brassicicola* and *R. solani* and this metabolism is a detoxification as both indole-3-carboxaldehyde (**47**) and indole-3-carboxylic acid (**48**) are less inhibitory than indole-3-methanol (**110**) against both pathogens. This oxidative metabolism of indolyl-3-methanol (**110**) to indole-3-carboxaldehyde (**47**) and indole-3-carboxylic acid (**48**) was reported in mammalian systems and in plants earlier but no such transformation has been reported in fungi (Agerbirk et al., 2009). The metabolic products of all glucosinolates and derivatives by all three pathogens are summarized in the Table 2.8.

Table 2.8 Metabolism of compounds (**40**, **65**, **66**, **86**, **87**, **90**, **102**, **103**, **107**, **110**, **113**, **159**, **170**, **185**, **202**, **228**, **233**, and **235**) by the plant fungal pathogens *Alternaria brassicicola*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* under continuous light.

Compound name	Metabolic products and rate of transformation		
	A. brassicicola	*R. solani*	*S. sclerotiorum*
Glucobrassicin (**86**)	No transformation	No transformation	No transformation
1-Methoxy glucobrassicin (**87**)	No transformation	No transformation	No transformation
4-Methoxy glucobrassicin (**90**)	No transformation	No transformation	No transformation
Benzyl glucosinolate (**66**)	Phenyl acetonitrile (**185**) and benzyl isothiocyanate (**170**); 100% in 12 h	No transformation	No transformation
Phenyl glucosinolate (**65**)	Benzonitrile (**235**); 100% in 72 h	No transformation	No transformation
Desulfo glucobrassicin (**159**)	No transformation	Indolyl-3-acetontirile (**40**); 50% in 24 h	Indolyl-3-acetontirile (**40**); 50% in 108 h
1-Methoxy desulfo glucobrassicin (**233**)	No transformation	1-Methoxyindolyl-3-acetontirile (**102**); 50% in 84 h	1-Methoxyindolyl-3-acetontirile (**102**); 50% in 72 h
Benzyldesulfo glucosinolate (**202**)	No transformation	No transformation	No transformation
Phenyldesulfo glucosinolate (**228**)	Benzonitrile (**235**); 50% in 60 h	No transformation	No transformation
Indoyl-3-acetonitrile (**40**)	Indolyl-3-carboxylic acid (**48**); 100% in 12 h	Indolyl-3-carboxylic acid (**48**); 75% in 5 days[a]	Indolyl-3-carboxylic acid (**48**); 100% in 24 h[a]

Compound name	Metabolic products and rate of transformation		
	A. brassicicola	*R. solani*	*S. sclerotiorum*
1-Methoxy indolyl-3-acetonitrile (**102**)	1-Methoxyindolyl-3-carboxylic acid (**241**); 50% in 20 h	1-Methoxyindolyl-3-carboxylic acid (**241**); 50% in 42 h	1-Methoxyindolyl-3-carboxylic acid (**241**); 50% in 54 h
4-Methoxy indolyl-3-acetonitrile (**103**)	4-Methoxyindolyl-3-carboxylic acid (**239**); 50% in 48 h	4-Methoxyindolyl-3-carboxylic acid (**239**); 50% in 14 h	4-Methoxyindolyl-3-carboxylic acid (**239**); 50% in 36 h
Phenyl acetonitrile (**185**)	Benzoic acid (**235**); 100% in 12 h	No transformation	Benzoic acid (**235**); 100% in 12 h
Benzonitrile (**235**)	No transformation	No transformation	Benzoic acid; 50% in 24 h
Indolyl-3-methanol (**110**)	Indolyl-3-carboxylic acid (**48**); 100% in12 h	Indolyl-3-carboxylic acid (**48**); 100% in 12 h	Unstable in acidic medium
Diindolyl-3-methane (**113**)	No transformation	No transformation	No transformation
Ascorbigen (**107**)	No transformation	No transformation	No transformation
Benzyl isothiocyanate (**170**)	Transformed to undetectable compound(s); 100% in 24 h	No transformation	No transformation

[a]Results from previous work, Pedras and Montaut, 2003.

Myrosinase activity was found in crude extract of *A. brassicicola*. Myrosinase activity of crude extract of *A. brassicicola* was tested against several glucosinolates including aliphatic, aryl and indolyl glucosinolates using sodium ascorbate. Like the myrosinases from fungal species of *Aspergillus* myrosinase of *A. brassicicola* also showed higher activity against aliphatic and aryl glucosinolates but relatively lower activity against indolyl glucosinolate.

2.3. Overall conclusion and future work

In this thesis, it has been shown that *B. cinerea* is able to circumvent phytoalexins of crucifers through metabolism and detoxification (Pedras et al., 2011b). Phytoalexins, strongly and moderately antifungal to *B. cinerea*, detoxified to products, which are less toxic to *B. cinerea*. Two very important cruciferous phytoalexins (**33** and **39**) was tested for antifungal activity and metabolism against plant pathogen *B. cinerea*. Camalexin (**39**) was found to be more antifungal than brassinin (**33**); while camalexin (**39**) was transformed via oxidative degradation, brassinin (**33**) was hydrolyzed to indoly-3-methanamine (**49**). Despite close similarity between two pathogens (*B. cinerea* and *S. sclerotiorum*) and presence of a similar enzyme of SsBGT1 in *B. cinerea*, none of the compounds was metabolized following pathways in *S. sclerotiorum*.

The first study of the direct interaction of various indole and aryl glucosinolates and derivatives with plant pathogenic fungi provided evidence that through their derivatives, glucosinolates play important roles in plant defense reactions (Pedras and Hossain, 2011). None of the glucosinolates or their desulfo derivatives showed antifungal activities but some of their derived metabolites showed low to very high antifungal activities. Among these metabolites, diindolyl-3-methane (**113**) showed highest antifungal activity, between two isothiocyanates benzyl isothiocyanate (**170**) showed higher inhibitory effect against generalist pathogens *R. solani* and *S. sclerotiorum* but none of them are significantly inhibitory against the specialist pathogen *A. brassicicola* at the highest concentration tested. Indolyl acetonitriles (**40**, **102**, and **103**) showed variable antifungal activities and ascorbigen (**107**) showed the lowest antifungal activity. Aryl glucosinolates (**65** and **66**) are metabolized by the specialist but not by the generalist, whereas indolylglucosinolates were not metabolized

any pathogen. Indolyl desulfoglucosinolates (**159** and **233**) were metabolized by two non-host selective pathogens (*R. solani* and *S. sclerotiorum*) to the corresponding carboxylic acid through acetonitriles and acetic acid derivatives, which is a detoxification process. Metabolism of indolyl-3-methanol (**110**) by *A. brassicicola* and *R.* solani to indolyl-3-carboxylic acid (**48**) is a detoxification process. A low but consistent myrosinase activity was found in a cruciferous fungal pathogen (*A. brassicicola*) for the first time. The cell-free extracts of mycelia of *A. brassicicola* displayed higher myrosinase activity for sinigrin (**131**), phenyl and benzyl glucosinolates (**65** and **66**), but lower activities for glucobrassicin (**86**) and 1-methoxyglucobrassicin (**87**); no myrosinase activity was detected in mycelia of either *R. solani* or *S. sclerotiorum.*

These results indicated that diindolyl-3- methane (**113**) was the most antifungal among indole glucosinolate derivatives and barred to metabolism of any of the tested fungal pathogens, thus could potentially increase plant resistance to diverse fungal pathogens if upregulated (Pedras and Hossain, 2011). Contrary to earlier results on the interaction of glucosinolates with insects, none of the glucosinolates appeared to have an impact on the growth of the plant fungal pathogens investigated in this work. Desulfoglucosinolates are important in the biosynthetic pathway of glucosinolates and many other phytoalexins, but are metabolized by pathogenic fungi. Prevention of metabolism of desulfoglucosinolates may help plant to produce more phytoalexins.

Future work

1. The cruciferous phytoalexin brassinin (**33**) is of great interest because of its biological activity and intermediacy in the biosynthetic pathway of other relevant phytoalexins such as cyclobrassinin (**35**), brassilexin (**45**), rutalexin (**46**), brassicanal A

(**38**), and spirobrassinin (**36**) (Pedras et al., 2003a). Therefore, it is expected that the inhibition of brassinin (**33**) detoxification will allow plants to accumulate all these phytoalexins. These accumulated phytoalexins are then expected to slow if not stop the growth of *B. cinerea.* Which can be achieved by: a) purification and characterization of enzymes involved in the metabolism of phytoalexins (**33** and **39**) by *B. cinerea* and b) design and synthesis of inhibitors of enzymes responsible for the metabolism of phytoalexins.

2. Desulfoglucobrassicins are precursors of glucobrassicins which are further precursors of many phytoalexins and phytoanticipins, but are metabolized by pathogenic fungi *R. solani* and *S. sclerotiorum*. Prevention of metabolism of desulfoglucosinolates may help plant to produce more phytoalexins and important phytoanticipins. Purification and characterization of enzymes involved in the metabolism of desulfoglucobrassicins by *R. solani* and *S. sclerotiorum* and design and synthesis of potential inhibitors of desulfoglucobrassicins may help plant defense against these pathogens.

3. Myrosinase activity was first time observed in a pathogenic fungus of crucifers. It will be interesting to compare myrosinases from *A. brassicicola* with myrosinases from plants. Separation, purification, and characterization of myrosinase(s) from the phytopathogenic fungus *A. brassicicola* can help to study the kinetic and substrate specificity studies using purified myrosinase(s) from *A. brassicicola* and compare the results with those of plant myrosinases.

CHAPTER 3 : EXPERIMENTAL

3.1. Chemicals and instrumentation

All solvents were HPLC grade and used as such, except for any solvents used in synthetic procedures (dried as necessary for each procedure). Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Flash column chromatography (FCC): silica gel, grade 60, 230-400 μm. Organic extracts were dried over Na_2SO_4 and the solvents were removed under vacuum using a rotary evaporator. HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190 - 600 nm), degasser, and a column, having an in-line filter. Elution method A: column Eclipse XDB-C18 (5 μm particle size silica, 150 mm × 4.6 mm i.d.), mobile phase 50% H_2O - 50% CH_3OH to 100% CH_3OH, for 25.0 min, linear gradient, and at a flow rate 0.75 mL/min; elution method B (indolyl glucosinolates and other polar metabolites): column Zorbax SB-C18 (3.5 μm particle size silica, 100 mm × 3.0 mm i.d.), equipped with an in-line filter, with the mobile phase H_2O (with 0.1% TFA) - CH_3OH (with 0.1% TFA) from 85:15 to 70:30 in 25 min, to 50:50 in 5 min, to 40:60 in 5 min and a flow rate of 0.40 mL/min; elution method C: column Eclipse XDB-C18 (5 μm particle size silica, 150 mm × 4.6 mm i.d.), mobile phase 90% H_2O - 10% CH_3OH to 50% CH_3OH, for 30.0 min, linear gradient, and at a flow rate 0.75 mL/min. HPLC-DAD-ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an auto sampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2440A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separation was carried out at room

temperature using an Eclipse XDB C-18 column (5 µm particle size silica, 150 mm × 4.6 mm i.d). The mobile phase consisted of a linear gradient of 0.2% formic acid in water and 0.2% formic acid in CH_3CN (75:25 to 25:75 in 35 min, to 0:100 in 5 min) and a flow rate of 1 mL/min. Data acquisition was carried out in positive and negative polarity modes in a single LC run. All other experimental conditions were as reported previously (Pedras et al., 2010).

3.1.1. Preparation of minimal media

A solution of glucose (15.0 g) in 700 mL of distilled water was mixed with solution 1 (100 mL) containing 31.2 g/L KNO_3, 7.5 g/L K_2HPO_4, 7.5 g/L KH_2PO_4, 1.0 g/L NaCl and 2.8 g/L asparagines plus solution 3 (1 mL) containing 0.39 g/L $ZnSO_4.7H_2O$, 0.08 g/L $CuSO_4.5H_2O$, 0.41 g/L $MnSO_4.4H_2O$, 0.018 g/L MoO_3 (85%), 0.54 g/L ferric citrate and 0.38 g/L $Na_2B_4O_7.10H_2O$. The mixture was diluted up to 900 mL using distilled water and autoclaved. Solution 2 (100 mL), containing 1.0 g/L $CaCl_2$. $7H_2O$ and 5.0 g/L $MgSO_4.7H_2O$ was prepared separately and autoclaved. After autoclaving, the two solutions were allowed to cool to room temperature before mixing them together. A sterile solution 4 (1 mL) containing 100 mg/L of thiamine was then mixed to obtain the minimal media (Pedras et al., 1997).

3.1.2. Preparation of fungal cultures

B. cinerea isolates UAMH 1784 and UAMH 1809 were obtained from University of Alberta Microfungus Collection and Herbarium (UAMH), Edmonton, AB, Canada. The fungal isolates were grown on potato dextrose agar (PDA) plates by

inoculating fungal mycelia on the plates and were incubated at 20±1 ºC under constant light. After 3-4 days fungal mycelia were cut from the edge of mycelia and transferred to Erlenmeyer flasks containing minimal media. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media were inoculated with six mycelial plugs (6 mm dia) and were incubated at 22 ± 1 ºC on a shaker at 120 rpm under constant light.

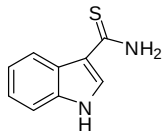
A. brassicicola (ATCC 96866) spores were grown on potato dextrose agar (PDA) plates (100 × 15 mm). PDA plates were then incubated under constant light at 22˚C ±1˚C. After two weeks, spores were collected and stored at -20˚C. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media were inoculated each time with eight-day old spores (10^6spores/100 mL) and incubated on a shaker at 120 rpm under constant light at a temperature of 22˚C ± 1˚C for 48 hours.

R. solani (AG 2-1) was obtained from AAFC-Saskatoon, SK, Canada. The fungal isolates were grown on potato dextrose agar (PDA) plates by inoculating fungal mycelia on the plates and were incubated at 20±1 ºC under constant light. After 3-4 days fungal mycelia were cut from the edge of mycelia and transferred to Erlenmeyer flask containing minimal media. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media were inoculated with six mycelial plugs (6 mm dia) and were incubated at 22±1 ºC on a shaker at 120 rpm under constant light.

Sclerotia of *S. sclerotiorum* (clone # 33) were obtained from AAFC, Saskatoon, SK, Canada. The fungal isolate was grown on potato dextrose agar (PDA) plates by inoculating one piece of sclerotia per plate and the plates were incubated at 20±1 ºC in the dark. After 2-3 days fungal mycelia were cut from the edge of mycelia and transferred to Erlenmeyer flask containing minimal media. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media were inoculated with six mycelial plugs (6 mm dia) and were incubated at 22±1 ºC on a shaker at 120 rpm under constant light.

3.2. Synthesis and characterization of compounds

3.2.1. 3-Indolethiocarboxamide (226)



226

3-Indolethiocarboxamide (**226**) was prepared from 3-indolecarboxynitrile (**225**) by modification of a previously published procedure (Gu et al., 1999), as follows. A mixture of 3-indolecarboxynitrile (**225**, 100.0 mg, 0.70 mmol) and thioacetamide (105.0 mg, 1.40 mmol) in 10% HCl–DMF solution (1.5 mL) was stirred at 95º C for 12 h. The reaction mixture was then neutralized with $NaHCO_3$ (sat. solution), extracted with EtOAc, the organic layer was dried and separated by FCC (silica gel, CH_2Cl_2–MeOH, 99:1) to afford 3-indolethiocarboxamide (**226**) (71.5 mg, 0.41 mmol, 59%) (Gu et al. 1999).

Mp = 151-152 °C

HPLC t_R = 4.6 min (method A)

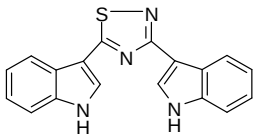
^{1}H NMR (DMSO-d_6) δ: 10.91 (1H, s), 8.08 (1H, s), 7.95 (1H, s), 7.75 (1H, d, J = 7.5 Hz), 7.22 (1H, d, J = 3.0 Hz), 6.56 (1H, d, J = 8.0 Hz), 6.29 (1H, dd, J = 7.0, 7.0 Hz), 6.26 (1H, dd, J = 7.0, 7.0 Hz)

^{13}C NMR (DMSO-d_6) δ: 193.6, 136.8, 128.1, 125.9, 122.0, 121.8, 120.7, 116.3, 112.0. HREI-MS m/z: calc. for $C_9H_8N_2S$ 176.0408, found 176.0409; m/z (%):176.0 $[M]^+$ (100), 160.0 (24), 143.1 (84), 142.1 (43), 116.0 (20)

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 215, 255, 318

FTIR (KBr, cm^{-1}) ν_{max}: 3190, 1621, 1527, 1442, 850.

3.2.2. 3,5-(3´, 3″-Bisindolyl)-1,2,4-thiadiazole (227)



227

3-Indolethiocarboxamide (**226**) (100.1 mg, 0.57 mmol) was added to a stirred suspension of IBX (166.0 mg, 0.63 mmol) in CH_3CN (2.0 mL). After completion of the reaction, the residue was filtered off and the solvent was evaporated under reduced pressure. The reaction mixture residue was dissolved in EtOAc (20 mL), washed with $NaHCO_3$ (sat. solution) and then H_2O. The organic layer was dried and concentrated under reduced pressure, and the residue was fractionated by FCC (silica gel, CH_2Cl_2–MeOH, 99:1) to yield 3,5-(3´,3″-bisindolyl)-1,2,4-thiadiazole (12) (40.5 mg, 0.13 mmol, 45%) and 3-indolecarboxynitrile (**225**) (16.1 mg, 0.11 mmol, 20%) (Patil et al., 2009).

Mp = 240-241 °C

HPLC t_R = 20.5 min (method A)

^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.26 (1 H, s), 10.85 (1H, s), 7.61 (1H, m), 7.56 (1H, m), 7.41 (2H, m), 6.71–6.65 (2H, m), 6.48–6.43 (2H, m), 6.38–6.34 (2H, m)

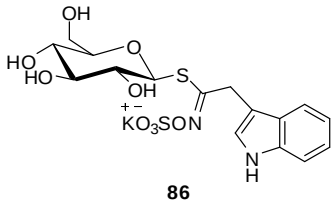
^{13}C NMR (125.8 MHz, DMSO-d_6) δ: 180.2, 170.1, 137.2, 137.1, 129.8, 129.2, 125.6, 124.8, 123.4, 122.6, 122.0, 121.6, 121.0, 120.6, 113.0, 112.5, 110.7, 108.1

HREI-MS m/z: calc. for $C_{18}H_{12}N_4S$: 316.0782, found 316.0787; m/z (%): 316.0 $[M]^+$ (81), 200.0 (19), 174.0 (100), 142.1 (57), 115.0 (18)

UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 220, 253, 318

FTIR (KBr, cm^{-1}) ν_{max}: 3100, 1531, 1338, 1236, 744.

3.2.3. Glucobrassicin (86)



86

Ammonium acetate (53.2 mg, 0.68 mmol) was added to a suspension of indole-3-carboxaldehyde (**47**, 200.1 mg, 1.38 mmol) in nitromethane (1.5 mL, 27.6 mmol). The mixture was vigorously stirred under reflux at 120–130 °C for 2 h, the reaction mixture was cooled to room temperature, diluted with water (20 mL), and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 265.8 mg of crude product, which was directly used in the next step without purification. To a stirred mixture of crude 3´-(1- nitrovinyl)indole (**133**, 259.7 mg, 1.38 mmol) in THF (7.0 mL) and MeOH (850 µL), $NaBH_4$ (206.5 mg, 5.50 mmol) was added in portions over a period of 30min. After 6 h, the remaining

$NaBH_4$ was quenched with aq HCl (1 M), the reaction mixture was filtered, the filtrate was dried and concentrated under reduced pressure to yield the crude reaction mixture. Separation over silica gel using CH_2Cl_2-hexane (4:1) as the eluent yielded a mixture of 3´-(1-nitroethyl)indole (**134**, 115.7 mg, 44%). Next, a solution of 3´-(1-nitroethyl)indole (**134**, 115.7 mg, 0.61 mmol) in MeOH (1.0 mL) was added to a stirred solution of MeONa (30 mg Na in anhydrous MeOH, 1.0 mL). After stirring for 30 min, the solvent was evaporated at room temperature, the reaction mixture was suspended in cooled DME (1.5 mL, -40 °C), and a solution of $SOCl_2$ (135 µL, 1.83 mmol) in DME (1 mL) was added drop wise via syringe under argon. After stirring for 1 h at -40 °C, the mixture was diluted with H_2O (5 mL), was concentrated (evaporation of most of the DME) under vacuum, and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layer was dried and concentrated under reduced pressure at room temperature to yield crude indolyl-3-acetohydroximoylchloride (**135**, 117.4 mg), which was directly used for the next step without further purification. 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranose (146.2 mg; 0.40 mmol) in dry CH_2Cl_2 (2 mL) and triethylamine (170 mL, 1.20 mmol) diluted with dry Et_2O (1mL) were successively added to the crude indolyl-3-acetohydroximoylchloride (**135**, 117.4 mg, 0.92 mmol) in Et_2O–CH_2Cl_2 (6 mL, 2 : 1 v/v) under argon. After stirring for 3 h at room temperature, the reaction mixture was acidified with H_2SO_4 (0.5 M), extracted with CH_2Cl_2 (3 × 40 mL), and the combined organic layer was dried and concentrated under reduced pressure to yield 210.1 mg of crude product. After column chromatography (silica gel, CH_2Cl_2–MeOH, 98:2), indolyl-3-tetraacetylglucoacetothiohydroximate (**136**) was obtained in 70% yield (150.1 mg). A solution of chlorosulfonic acid (325 µL, 2.80 mmol) in dry diethyl ether (1.2 mL) was added over a period of 30 min to a cooled and stirred solution of **136** (150.0 mg; 0.28 mmol) in dry pyridine (1.0 mL) and dry CH_2Cl_2 (1.2 mL) under argon. After stirring for 24 h at room temperature under argon, the reaction mixture was

diluted with an aq. $KHCO_3$ solution (100 mg in 2.0 mL), stirred for another hour, and extracted with $CHCl_3$ (3 × 50 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 145.2 mg of crude reaction product. Chromatography over silica gel (CH_2Cl_2–MeOH, 95: 5, v/v) afforded tetraacetylated glucobrassicin (**137**, 119.1 mg) in 65% yield. Hydrolysis of tetraacetylated glucobrassicin (**137**, 56.0 mg, 0.085 mmol) in anhydrous MeOH (2.0 mL) plus KOMe (ca. 1 M) to obtain pH 8, under argon at room temperature for 24 h, followed by neutralization with AcOH and concentration to dryness yielded 92.9 mg of glucobrassicin (**86**) containing KOAc.

Mp = 154-155 °C

HPLC t_R = 10.0 min (method B)

^{1}H NMR (500 MHz, D_2O) δ: 7.74 (1 H, d, J = 8.0 Hz), 7.51 (1 H, d, 8.0 Hz), 7.32 (1 H, s), 7.25 (1H, dd, J = 7.5, 7.5 Hz), 7.18 (1H, dd, J = 7.5, 7.5 Hz), 4.78 (1 H, d, J = 10.0 Hz), 4.26 (1H, d, J = 16.5 Hz), 4.13 (1H, d, J = 16.5 Hz), 3.53 (2H, d, J = 3.5 Hz), 3.31 (1H, dd, J = 9.5, 9.5 Hz), 3.25 (1H, dd, J = 9.5, 9.0 Hz), 3.16 (1H, dd, J = 9.0, 9.0 Hz), 2.92–2.88 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 163.1, 136.2, 126.1, 124.0, 122.2, 119.6, 118.4, 112.0, 108.1, 81.4, 79.8, 76.8, 71.7, 68.6, 60.1, 29.3

HR-ESI-MS m/z calc. for $C_{16}H_{19}N_2O_9S_2$: 447.0537; found 447.0547 $[M - 1]^-$.

3.2.4. 1-Methoxyglucobrassicin (87)

A solution of $Na_2WO_4.2H_2O$ (530.4 mg, 1.60 mmol) in water was added to the solution of indoline (**249**, 950 µL, 8.40 mmol) in MeOH (80 mL) with stirring. The

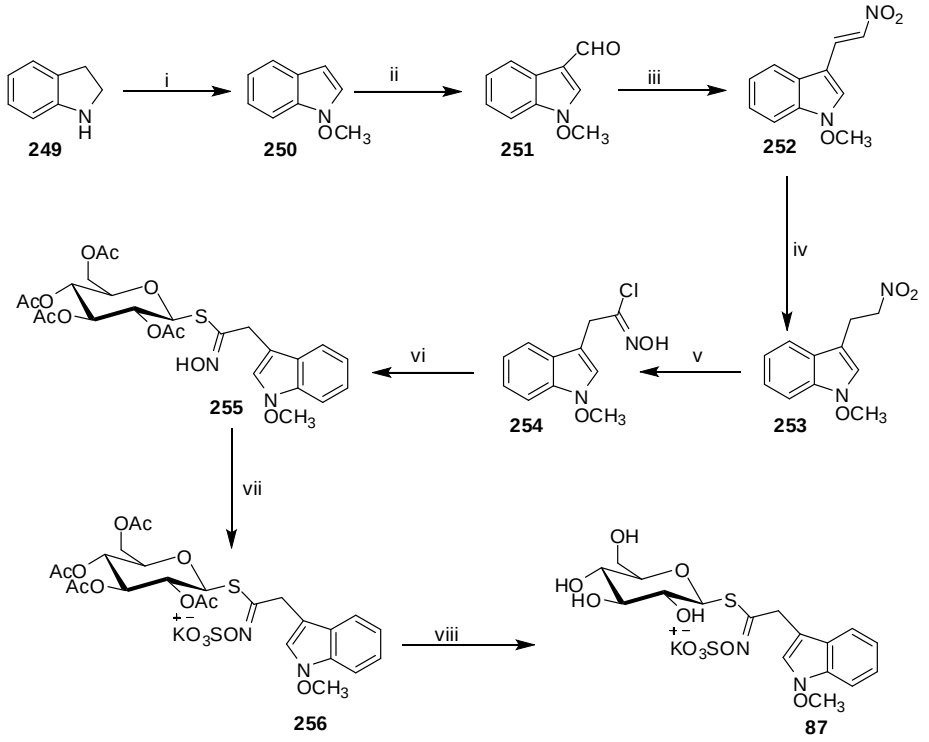
mixture was cooled to -20 ºC using NaCl-ice system. During 30 minutes a solution of 30% H_2O_2 (10 mL, 97.7 mmol) in MeOH (20 mL) was added slowly to the reaction mixture. The stirring was continued for further 10 minutes and then solid K_2CO_3 (10.1 g, 73.0 mmol) and dimethyl sulphate (2.3 mL, 25.4 mmol) were added with vigorous stirring. The mixture was stirred for further 1.5 hour at r.t., was poured into water (100 mL) and extracted with Et_2O (2 × 150 mL). After drying (Na_2SO_4) and evaporation of solvent, the greenish oily residue was column chromatographed on silica gel (CH_2Cl_2: hexane; 1:1) to yield 1-methoxyindole (**250**) (618.1 mg, 50%) (Kawasaki et al., 1991).

To the solution of 1-methoxyindole (**250**) (555.4 mg, 3.80 mmol) in DMF (1.0 mL) was added distilled $POCl_3$ (380 µL, 4.10 mmol). After stirring at room temperature for 1 h, the mixture was basified with 5 M NaOH, and then boiled for 5 minutes. The solution was extracted with Et_2O (2 × 10 mL), the organic phase was dried over Na_2SO_4 and concentrated to dryness to yield 1-methoxyindole-3-carboxaldehyde (**251,** 530.0 mg, 80%) (Pedras and Zaharia, 2000).

Ammonium acetate (88.1 mg, 1.14 mmol) was added to a suspension of 1-methoxyindole-3-carboxaldehyde (**251**, 400.3 mg, 2.28 mmol) in nitromethane (1.9 mL). The mixture was vigorously stirred under reflux at 120–130 °C for 2 h, the reaction mixture was cooled to room temperature, diluted with water (20 mL), and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 390 mg of crude product, which was directly used in the next step without purification. To a stirred mixture of crude 1-methoxy-3´-(1- nitrovinyl)indole (**252**, 496.4 mg, 2.28 mmol) in $CHCl_3$ (26 mL) and i-PrOH (7.5 mL), SiO_2 (4.4 g), $NaBH_4$ (344.0 mg, 9.10 mmol) was added in portions over a period of 30 min. After 6 h, the remaining $NaBH_4$ was quenched with aq HCl (1 M), the reaction mixture was filtered, the filtrate was dried and concentrated under

reduced pressure to yield the crude reaction mixture. Separation over silica gel using CH_2Cl_2–hexane (4:1) as the eluent yielded a mixture of 1-methoxy-3´-(1-nitroethyl)indole (**253**, 200.5 mg, 40%). Next, a solution of 1-methoxy-3´-(1-nitroethyl) indole (**253**, 208.5 mg, 0.95 mmol) in MeOH (2.0 mL) was added to a stirred solution of MeONa (30 mg Na in anhydrous MeOH, 1.0 mL). After stirring for 30 min, the solvent was evaporated at room temperature, the reaction mixture was suspended in cooled DME (2 mL, -40 °C), and a solution of $SOCl_2$ (170 µL, 2.37 mmol) in DME (1.0 mL) was added drop wise via syringe under argon. After stirring for 1 h at -40 °C, the mixture was diluted with H_2O (5 mL), was concentrated (evaporation of most of the DME) under vacuum, and extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layer was dried and concentrated under reduced pressure at room temperature to yield crude 1-methoxyindolyl-3-acetohydroximoylchloride (**254**, 230.5 mg,), which was directly used for the next step without further purification. 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranose (224.3 mg, 0.67 mmol) in dry CH_2Cl_2 (2.0 mL) and triethylamine (250 µL, 1.82 mmol) were successively added to the crude 1-methoxyindolyl-3-acetohydroximoylchloride (**254**, 226.6 mg, 0.95 mmol) in Et_2O–CH_2Cl_2 (6.0 mL, 2 : 1 v/v) under argon. After stirring for 3 h at room temperature, the reaction mixture was acidified with H_2SO_4 (0.5 M), extracted with CH_2Cl_2 (3 × 40 mL), and the combined organic layer was dried and concentrated under reduced pressure to yield 145.0 mg of crude product **255**. After column chromatography (silica gel, CH_2Cl_2–MeOH, 98:2), 1-methoxyindolyl-3-tetraacetylglucoacetothiohydroximate (**255**) was obtained in 32% yield (111.2 mg). A solution of chlorosulfonic acid (110 µL, 1.6 mmol) in dry diethyl ether (750 µL) was added over a period of 30 min to a cooled and stirred solution of **255** (99.1 mg, 0.16 mmol) in dry pyridine (600 µL) and dry CH_2Cl_2 (750 µL) under argon. After stirring for 24 h at room temperature under argon, the reaction mixture was diluted with an aq. $KHCO_3$ solution (0.1 g in 2.0 mL),

stirred for another hour, and extracted with $CHCl_3$ (3 × 30 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 130 mg of crude reaction product. Chromatography over silica gel (CH_2Cl_2–MeOH, 95: 5, v/v) afforded tetraacetyl 1-methoxyglucobrassicin (**256**, 72.3 mg) in 66% yield. Hydrolysis of tetraacetyl 1-methoxyglucobrassicin (**256**, 34.0 mg; 0.049 mmol) in anhydrous MeOH (1.5 mL) plus KOMe (1 M) to obtain pH 11, under argon at room temperature for 2 h, followed by neutralization with AcOH and concentration to dryness yielded 24.5 mg of 1-methoxyglucobrassicin (**87**) containing KOAc in 96% yield.



Scheme 3.1 Synthesis of 1-methoxyglucobrassicin (**87**). Reagents: (i) $Na_2WO_4.2H_2O$, 30% H_2O_2; (ii) Me_2SO_4, K_2CO_3, 50%; (iii) $POCl_3$, DMF; NaOH, 80%; (iv) CH_3NO_2, ammonium acetate, 120 -130º C; (v) $NaBH_4$,THF-MeOH, 40%; (vi) NaOMe-MeOH, $SOCl_2$, DME, -40 °C; iv. Thioglucose, Et_3N, Et_2O/CH_2Cl_2, 32%; (vii) HSO_3Cl, pyridine, $KHCO_3$, 66%; (viii) MeOK, MeOH, 96%. (Cassel et al. , 1998).

Mp = 142-143 °C

HPLC t_R = 16.1 min (method B)

^{1}H NMR (500 MHz, D_2O) δ: 7.75 (1 H, d, *J* = 8.0 Hz), 7.56 (1H, d, *J* = 8.5 Hz), 7.51 (1H, s), 7.35 (1H, dd, *J* = 8.0, 7.5 Hz), 7.22 (1H, dd, *J* = 7.5, 7.5 Hz), 4.78 (1H, d, *J* = 10.0 Hz), 4.22 (1H, d, *J* = 17.0 Hz), 4.14 (1H, d, *J* = 17.0 Hz), 4.08 (3H, s), 3.55-3.49 (2H, m), 3.32 (1H, dd, *J* = 9.5, 9.5 Hz), 3.27 (1H, dd, *J* = 10.0, 9.0 Hz), 3.17 (1H, dd, *J* = 9.0, 9.0 Hz), 2.97–2.94 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 162.6, 132.2, 123.1, 122.7, 122.6, 120.4, 118.9, 108.8, 105.3, 81.5, 79.9, 76.8, 71.7, 68.6, 66.0, 60.1, 29.0

HR-ESI-MS m/z calc. for $C_{17}H_{21}N_2O_{10}S_2$: 477.0643; found 477.0641 $[M - 1]^-$.

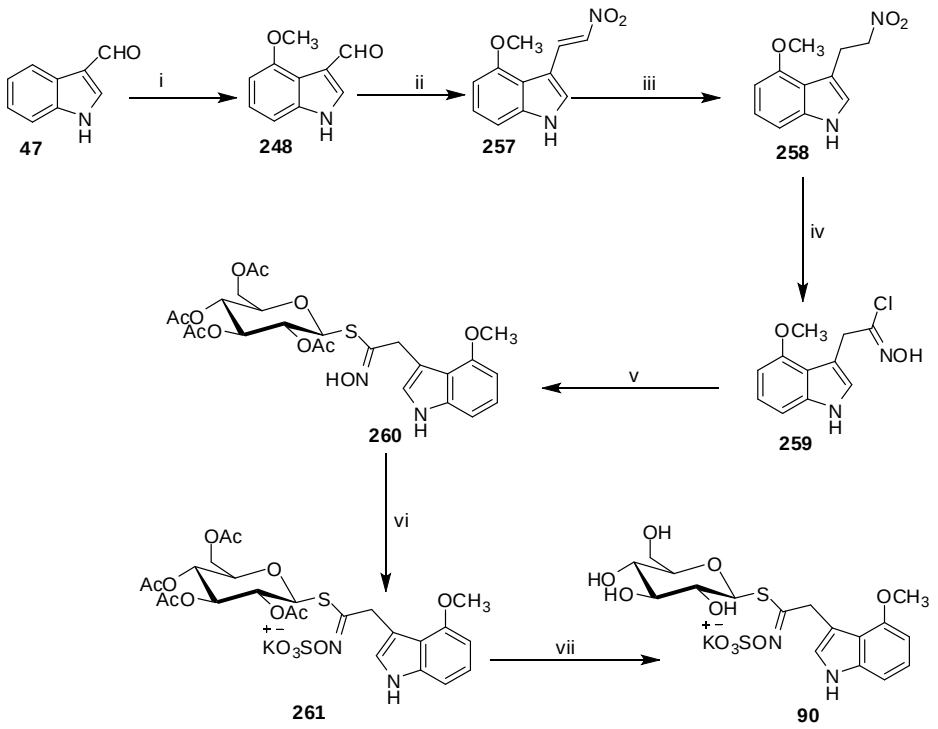
3.2.5. 4-Methoxyglucobrassicin (90)

Indole-3-carboxaldehyde (**47**, 50.0 mg, 0.35 mmol) was added to a solution of thallium trifluoroacetate (281.1 mg, 0.52 mmol) in TFA (600 µL), and the mixture was stirred at 30 °C for 3h. After evaporation of the solvent under reduced pressure, I_2 (263.2 mg, 1.03 mmol), CuI (262.5 mg, 1.38 mmol), and DMF (2.5 mL) were added to the residue. After stirring the reaction mixture at r.t. for 1h, $NaOCH_3$ (15% w/v, 3 mL) was added and the stirring continue at 100-110 °C for 3 h. The reaction was cooled to room temperature, diluted with CH_2Cl_2-MeOH (95:5) and filtered through celite. The filtrate was washed with water and brine, dried, and concentrated under reduced pressure. Purification of the product by FCC over silica using EtOAc: hexane (3:1) as the eluent yielded pure 4-methoxyindole-3-carboxaldehyde (**248**, 32.5 mg, 76%).

Ammonium acetate (67.5 mg, 0.88 mmol) was added to a suspension of 4-methoxyindole-3-carboxaldehyde (**248**, 306.1 mg, 1.75 mmol) in nitromethane (1.9

mL). The mixture was vigorously stirred under reflux at 120–130 °C for 2 h, the reaction mixture was cooled to room temperature, diluted with water (20 mL), and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 390 mg of crude product, which was directly used in the next step without purification. To a stirred mixture of crude 4-methoxy-3´-(1- nitrovinyl)indole (**257**, 381 mg, 1.75 mmol) in $CHCl_3$ (20 mL) and i-PrOH (5.8 mL), SiO_2 (3.4 g), $NaBH_4$ (264 mg, 7.0 mmol) was added in portions over a period of 30 min. After 6 h, the remaining $NaBH_4$ was quenched with aq HCl (1 M), the reaction mixture was filtered; the filtrate was dried and concentrated under reduced pressure to yield the crude reaction mixture. Separation over silica gel using CH_2Cl_2–hexane (4:1) as the eluent yielded a mixture of 4-methoxy-3´-(1-nitroethyl)indole (**258**, 170.1 mg, 44%). Next, a solution of 4-methoxy-3´-(1-nitroethyl)indole (**258**, 133.1 mg, 0.61 mmol) in MeOH (2.0 mL) was added to a stirred solution of MeONa (30 mg Na in anhydrous MeOH, 1.0 mL). After stirring for 30 min, the solvent was evaporated at room temperature, the reaction mixture was suspended in cooled DME (2.0 mL, -40 °C), and a solution of $SOCl_2$ (130 µL, 1.82 mmol) in DME (1.0 mL) was added drop wise via syringe under argon. After stirring for 1 h at -40 °C, the mixture was diluted with H_2O (5 mL), was concentrated under vacuum (evaporation of most of the DME), and extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layer was dried and concentrated under reduced pressure at room temperature to yield crude 4-methoxyindolyl-3-acetohydroximoylchloride (**259**, 152.3 mg, 0.61 mmol), which was directly used for the next step without further purification. 2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranose (220.2 mg, 0.0.61 mmol) in dry CH_2Cl_2 (2 mL) and triethylamine (250 µL, 1.82 mmol) diluted with dry Et_2O (1mL) were successively added to the crude 4-methoxyindolyl-3-acetohydroximoylchloride (**259**, 144.3 mg, 0.61 mmol) in Et_2O–CH_2Cl_2 (6 mL, 2 : 1 v/v) under argon. After stirring for 3 h at room

temperature, the reaction mixture was acidified with H_2SO_4 (0.5 M), extracted with CH_2Cl_2 (3 × 40 mL), and the combined organic layer was dried and concentrated under reduced pressure to yield 245.0 mg of crude product **260**. After column chromatography (silica gel, CH_2Cl_2–MeOH, 98:2), 4-methoxyindolyl-3-tetraacetylglucoacetothiohydroximate (**260**) was obtained in 32% yield (111.2 mg). A solution of chlorosulfonic acid (120 µL, 1.8 mmol) in dry diethyl ether (630 µL) was added over a period of 30 min to a cooled and stirred solution of **260** (99.2 mg; 0.18 mmol) in dry pyridine (600 µL) and dry CH_2Cl_2 (630 µL) under argon. After stirring for 24 h at room temperature under argon, the reaction mixture was diluted with an aq. $KHCO_3$ solution (100.0 mg in 2.0 mL), stirred for another hour, and extracted with $CHCl_3$ (3 × 50 mL). The combined organic layer was dried and concentrated under reduced pressure to yield 130 mg of crude reaction product. Chromatography over silica gel (CH_2Cl_2–MeOH, 95: 5, v/v) afforded tetraacetyl 4-methoxyglucobrassicin (**261**, 81.5 mg) in 66% yield. Hydrolysis of tetraacetyl 4-methoxyglucobrassicin (**261**, 81.5 mg; 0.11 mmol) in anhydrous MeOH (2 mL) plus KOMe (1 M) to obtain pH 11, under argon at room temperature for 2 h, followed by neutralization with AcOH and concentration to dryness yielded 62.5 mg of 4-methoxyglucobrassicin (**90**) containing traces of KOAc.



Scheme 3.2 Synthesis of 4-methoxyglucobrassicin (**90**). Reagents: (i) $Tl(CF_3COO)_3$-TFA, I_2, CuI, DMF, r.t., NaOMe, reflux, 76%; (ii) CH_3NO_2, ammonium acetate, 120 - 130 ºC; (iii) $NaBH_4$,THF-MeOH, 40%; (iv) NaOMe-MeOH, $SOCl_2$, DME, -40 °C; (v) Thioglucose, Et_3N, Et_2O/CH_2Cl_2, 32%; (vi) HSO_3Cl, pyridine, $KHCO_3$, 66%; (vii) MeOK, MeOH, Quantitative. (Cassel et al., 1998).

Mp = 146-147 °C

HPLC t_R = 14.1 min (method B)

^{1}H NMR (500 MHz, D_2O) δ: 7.16-7.10 (3H, m), 6.63 (1 H, d, J = 7.5 Hz), 4.84 (1H, d, J = 10.0 Hz), 4.42 (1H, d, J = 16.5 Hz), 4.35 (1H, d, J = 16.5 Hz), 3.92 (3H, s), 3.52 (1H, dd, J = 12.5, 2.5 Hz), 3.41 (1H, d, J = 12.0 Hz), 3.35 (1H, dd, J = 9.5, 9.5 Hz), 3.27 (1H, dd, J = 9.5, 9.5 Hz), 3.13 (1H, dd, J = 9.0, 9.0 Hz), 2.73 (1H, d, J = 9.5 Hz)

^{13}C NMR (125.8 MHz, D_2O) δ: 164.1, 153.8, 137.8, 123.1, 122.9, 115.8, 108.3, 105.5, 100.0, 81.8, 79.5, 77.0, 71.6, 68.2, 59.8, 30.1

HR-ESI-MS m/z calc. for $C_{17}H_{21}N_2O_{10}S_2$: 477.0643; found 477.0631 $[M - 1]^-$.

3.2.6. Phenylglucosinolate (65)

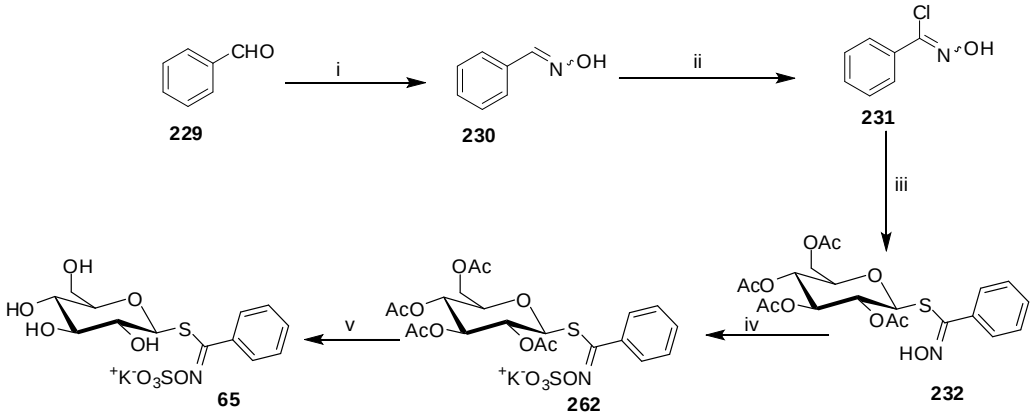
Benzaldehyde (**229**, 520 µL, 4.70 mmol) was dissolved in EtOH (5 mL) and a solution of $NH_2OH.HCl$ (340.0 mg, 4.90 mmol) and Na_2CO_3 (280.0 mg, 2.60 mmol) in water (5 mL) was added. After stirring at 60 ºC for 4 hours, the reaction mixture was diluted with water and extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give benzaldehyde oxime (**230**) (mixture of E and Z isomer) (99%).

A solution of benzaldehyde oxime (**230**, 200.2 mg, 1.60 mmol) in 3.0 mL DMF and 100 µL pyridine was cooled at 0º C in an ice bath and NCS (213.6 mg, 1.60 mmol) was added in portions over a period of 45 minutes. Upon completion, the ice bath was removed and continues stirring for 2 hours. 1-Thio-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (270.0 mg, 0.74 mmol) dissolved in DMF (4.0 mL) was added to the reaction mixture followed by triethylamine (200 µL) and stirred for another 18 h at room temperature. After 18h the reaction mixture was diluted with diethyl ether followed by 10 mL of 0.5 M H_2SO_4 and extracted with ethyl acetate (3 × 40 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a crude reaction mixture. The crude reaction mixture was separated by FCC over silica using hexane: ethyl acetate (1:1) as the eluent to give pure 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate (**232**) in 60% yield.

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate (**232**, 460.2 mg, 0.95 mmol) in dry pyridine (3.0 mL) and dry CH_2Cl_2 (3.5 mL) under inert atmosphere a solution of chlorosulfonic acid (630 µL; 9.50 mmol) diluted with dry diethyl ether (3.5 mL) was added over a period of 30 minutes. After stirring at room temperature for 24 h under argon atm. the reaction

mixture was then treated with $KHCO_3$ solution (100.0 mg in 1.3 mL H_2O) and stirred for another hour and extracted with $CHCl_3$ (3 × 50 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give 400.5 mg of crude reaction mixture which was then purified by FCC over silica using 5% methanol in CH_2Cl_2 as the eluent to give 322.3 mg of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate-N-sulfate (**262**) in 56.2% yield.

To a stirred solution of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl phenylthiohydroximate-N-sulfate (**262**, 122.1 mg, 0.20 mmol) in anhydrous methanol (8.5 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2 h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give phenylglucosinolate in 85% yield (**65**).



Scheme 3.3 Synthesis of phenylglucosinolate (**65**). Reagents: (i) $NH_2OH.HCl$, Na_2CO_3, H_2O, EtOH, 90%; (ii) NCS, Pyridine, DMF,; (iii) Thioglucose tetraacetate, Et_3N, 60%; (iv) HSO_3Cl, pyridine, 56%; (v) MeOK, MeOH, 85% (Mays et al., 2008).

Mp = 139-140 °C

HPLC t_R = 2.9 min (Method B)

^{1}H NMR (500 MHz, D_2O) δ: 7.52-7.28 (5H, m), 4.17 (1 H, d, J = 10.0 Hz), 3.57 (1H, d, J = 13.0 Hz), 3.48 (1H, dd, J = 13.0, 5.0 Hz) , 3.34 (1H, dd, J = 10.0, 9.0 Hz), 3.25 (1H, dd, J = 9.5, 9.5 Hz), 3.13 (1H, dd, J = 9.0, 9.0 Hz), 2.62-2.59 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 164.0, 131.0, 130.3, 129.0, 128.8, 83.5, 79.9, 77.0, 71.6, 68.7, 60.1

HR-ESI-MS mz calc. for $C_{13}H_{16}NO_9S_2$: 394.0271, found 394.0279 $[M-1]^-$.

3.2.7. Benzylglucosinolate (66)

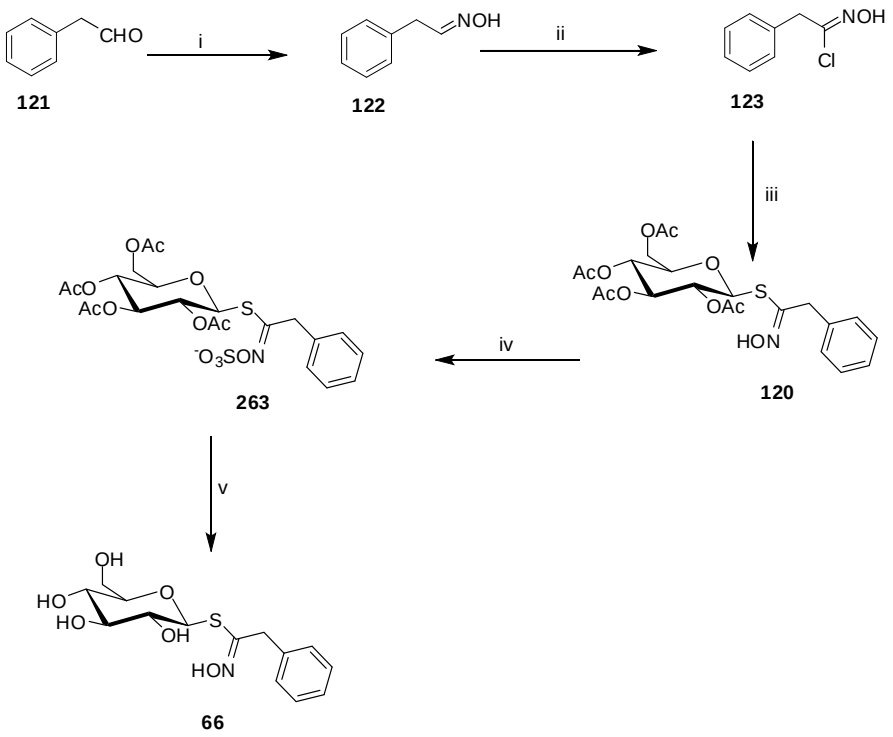
Phenyl acetaldehyde (**121**, 500.3 mg, 4.20 mmol) was dissolved in EtOH (15 mL) and a solution of $NH_2OH.HCl$ (580.0 mg, 8.30 mmol) and Na_2CO_3 (460.2 mg, 4.60 mmol) in water (8 mL) was added. After stirring at 60 ºC for 1 h, the reaction mixture was diluted with water and extracted with Et_2O (2 × 40 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give phenyl acetaldehyde oxime (**122**) The crude reaction mix was separated by FCC over silica using hexane : EA (4:1) to give mixture of E and Z isomer in 70% yield.

A solution of phenyl acetaldehyde oxime (**122** , 200.0 mg, 1.50 mmol) in 4 mL DMF was cooled at 0 ºC in an ice bath and NCS (198.2 mg; 1.50 mmol) was added in portions over a period of 45 minutes. Upon completion, the ice bath was removed and continued stirring for 2 hours. 1-Thio-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (270.0 mg; 0.74 mmol) dissolved in DMF (4 mL) was added to the reaction mixture followed by triethylamine (0.20 mL) and stirred for another 18 h at room temperature. After 18 h the reaction mixture was diluted with diethyl ether followed by 10 mL of 0.5 M H_2SO_4 and extracted with ethyl acetate (3 × 40 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a crude reaction mixture.

The crude reaction mixture was separated by FCC over silica using hexane: ethyl acetate (1:1) as the eluent to give pure 2,3,4,6-tetra-O-acetyl-β-glucopyranosylbenzylthiohydroximate (**123**) in 78% yield.

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylbenzylthiohydroximate (**123**, 130.0 mg, 0.27 mmol) in dry pyridine (1.0 mL) and dry CH_2Cl_2 (1.0 mL) under inert atmosphere a solution of chlorosulfonic acid (180 µL, 2.70 mmol) diluted with dry diethyl ether (1.0 mL) was added over a period of 30 minutes. After stirring at room temperature for 24 h under argon atm. the reaction mixture was then treated with $KHCO_3$ solution (0.1 g in 1.3 mL H_2O), stirred for another hour, and extracted with $CHCl_3$ (3 × 30 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give 100.8 mg of crude reaction mixture which was then purified by FCC over silica using 5% methanol in CH_2Cl_2 as the eluent to give 50.1 mg of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate-N-sulfate (**263**) in 55% yield.

To a stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylbenzylthiohydroximate-N-sulfate (**263**, 47.0 mg, 0.07 mmol) in anhydrous methanol (2.9 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give quantitative yield of benzylglucosinolate (**66**).



Scheme 3.4 Synthesis of benzylglucosinolate (**66**). Reagents: (i) $NH_2OH.HCl$, Na_2CO_3, H_2O, EtOH, 70%; (ii) NCS, Pyridine, DMF,; (iii) Thioglucose, Et_3N, 78%; (iv) HSO_3Cl, pyridine, 55%; (v) MeOK, MeOH, 100% (Mays et al., 2008).

Mp = 134-135 °C

HPLC t_R = 3.2 min (Method C)

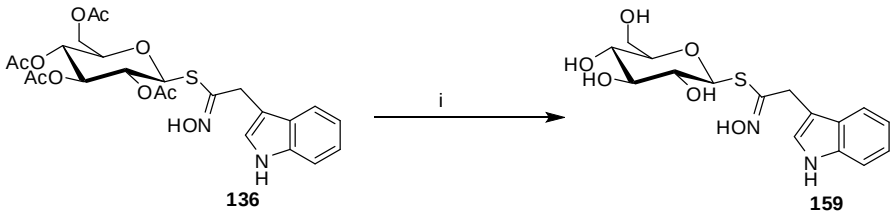
^{1}H NMR (500 MHz, D_2O) δ: 7.44-7.35 (5H, m), 4.70-4.68 (1 H, m), 4.13 (2H, br, s), 3.66-3.60 (2H, m), 3.40-3.36 (1H, m), 3.33-3.29 (2H, m), 3.25-3.22 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 162.7, 135.0, 129.2, 128.0, 127.6, 81.3, 79.8, 76.9, 71.8, 68.8, 60.3, 38.2

HR-ESI-MS m/z calc. for $C_{14}H_{18}NO_9S_2$: 408.0428, found 408.0435 $[M-1]^-$.

3.2.8. Desulfoglucobrassicin (159)

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylindolylthio hydroximate (**136**, 510.0 mg, 0.95 mmol) in anhydrous methanol (8.5 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2 h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give desulfoglucobrassicin (297.5 mg) in 85% yield (**159**) (Robertson and Botting, 1999).



Scheme 3.5 Synthesis of desulfoglucobrassicin (**159**). Reagents: (i) MeOK, MeOH, 85%.

Mp = 122-123 °C

HPLC t_R = 14.0 min (method B)

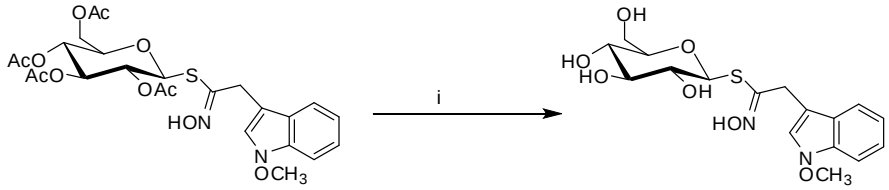
^{1}H NMR (500 MHz, D_2O) δ: 7.66 (1 H, d, *J* = 8.0 Hz), 7.49 (1 H, d, *J* = 8.0 Hz), 7.25-7.23 (2 H, m), 7.16 (1H, dd, *J* = 7.5, 7.5 Hz), 4.77 (1 H, d, *J* = 10.0 Hz), 4.13 (1H, d, *J* = 16.5 Hz),), 4.05 (1H, d, *J* = 16.5 Hz), 3.57-3.51 (2H, m), 3.31 (1H, dd, *J* = 9.5, 9.5 Hz), 3.25 (1H, dd, *J* = 9.5, 9.0 Hz), 3.17 (1H, dd, *J* = 9.0, 9.0 Hz), 2.94–2.92 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 154.6, 136.2, 126.2, 123.8, 122.1, 119.5, 118.4, 111.9, 109.2, 81.1, 79.7, 76.9, 72.0, 68.7, 60.2, 28.8

HR-ESI-MS m/z calc. for $C_{16}H_{19}N_2O_6S$: 367.0969; found 367.0975 $[M - 1]^-$.

3.2.9. 1-Methoxydesulfoglucobrassicin (233)

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosyl-1-methoxyindolylthio hydroximate (**255**, 311.6 mg; 0.55 mmol) in anhydrous methanol (8.5 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2 h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give 1-methoxydesufoglucobrassicin (197.1 mg) in 90% yield (**233**) (Robertson and Botting, 1999).



255 **233**

Scheme 3.6 Synthesis of 1-methoxydesulfoglucobrassicin (**233**). Reagents: (i) MeOK, MeOH, 90%.

Mp = 119-120 °C

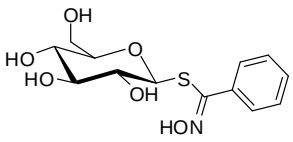
HPLC t_R = 23.6 min (method B)

^{1}H NMR (500 MHz, D_2O) δ: 7.56 (1 H, d, *J* = 8.0 Hz), 7.44 (1 H, d, *J* = 8.0 Hz), 7.31 (1 H, s), 7.23 (1H, dd, *J* = 8.0, 7.5 Hz), 7.09 (1H, dd, *J* = 7.5, 7.5 Hz), 4.68 (1 H, s), 3.97 (3H, s), 4.00 (1H, d, *J* = 17.0 Hz), 3.93 (1H, d, *J* = 17.0 Hz), 3.46 (2H, d, *J* = 3.5 Hz), 3.24 (1H, dd, *J* = 9.5, 9.5 Hz), 3.19 (1H, dd, *J* = 9.5, 9.0 Hz), 3.11 (1H, dd, *J* = 9.0, 9.0 Hz), 2.92–2.89 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 154.5, 132.3, 123.0, 122.8, 122.5, 120.3, 118.9, 108.8, 106.2, 81.2, 79.8, 76.9, 72.0, 68.7, 65.9, 60.2, 28.6

HR-ESI-MS m/z calc. for $C_{17}H_{21}N_2O_7S$: 397.1074; found 397.1083 $[M - 1]^-$.

3.2.10. Phenyldesulfoglucosinolate (228)



228

Benzaldehyde (**229**, 520 µL, 4.70 mmol) was dissolved in EtOH (5 mL) and a solution of $NH_2OH.HCl$ (340.0 mg, 4.90 mmol) and Na_2CO_3 (280.0 mg, 2.60 mmol) in water (5 mL) was added. After stirring at 60 ºC for 4 hours, the reaction mixture was diluted with water and extracted with CH_2Cl_2 (2 × 50 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give benzaldehyde oxime (**230**) (mixture of E and Z isomer) (99%).

A solution of benzaldehyde oxime (**230**, 200.2 mg, 1.60 mmol) in 3.0 mL DMF and 100 µL pyridine was cooled at 0º C in an ice bath and NCS (213.6 mg, 1.60 mmol) was added in portions over a period of 45 minutes. Upon completion the ice bath was removed and continues stirring for 2 hours. 1-Thio-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (270.0 mg, 0.74 mmol) dissolved in DMF (4.0 mL) was added to the reaction mixture followed by triethylamine (200 µL) and stirred for another 18 h at room temperature. After 18h the reaction mixture was diluted with diethyl ether followed by 10 mL of 0.5 M H_2SO_4 and extracted with ethyl acetate (3 × 40 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced

pressure to give a crude reaction mixture. The crude reaction mixture was separated by FCC over silica using hexane: ethyl acetate (1:1) as the eluent to give pure 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate (**232**) in 60% yield.

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylphenylthiohydroximate (**232**, 460.2 mg; 0.95 mmol) in anhydrous methanol (8.5 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2 h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give phenyldesulfoglucosinolate (**228**) in 85% yield.

Mp = 105-106 °C

HPLC t_R = 11.4 min (Method C)

$[\alpha]_D$ = -21.9 (c 0.187, H_2O)

UV (HPLC, MeOH-H_2O) λ_{max} (nm): 210

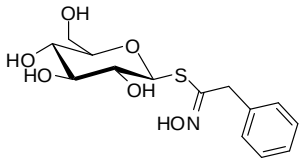
^{1}H NMR (500 MHz, D_2O) δ: 7.55-7.50 (5H, m), 4.25 (1 H, d, J = 10.0 Hz), 3.65 (1H, dd, J = 13.0, 2.5 Hz), 3.56 (1H, dd, J = 13.0 , 5.0 Hz) , 3.41 (1H, dd, J = 10.0, 10.0 Hz), 3.33 (1H, dd, J = 10.0, 9.5 Hz), 3.22 (1H, dd, J = 9.0, 9.0 Hz), 2.70-2.68 (1H, m)

^{13}C NMR (125.8 MHz, D_2O) δ: 156.0, 131.7, 130.4, 129.0, 128.7, 83.1, 79.9, 77.2, 71.9, 68.9, 60.3

HR-ESI-MS mz calc. for $C_{13}H_{16}NO_6S$: 314.0692, found 314.0686 $[M-1]^-$

FTIR (KBr, cm^{-1}) ν_{max}: 3360, 2887, 1613, 1560, 1494, 1410, 1340, 1050, 983, 703.

3.2.11. Benzyldesulfoglucosinolate (202)



202

Phenyl acetaldehyde (**121**, 500.3 mg, 4.20 mmol) was dissolved in EtOH (15 mL) and a solution of $NH_2OH.HCl$ (580.0 mg, 8.30 mmol) and Na_2CO_3 (460.2 mg, 4.60 mmol) in water (8 mL) was added. After stirring at 60 ºC for 1 h, the reaction mixture was diluted with water and extracted with Et_2O (2 × 40 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give phenyl acetaldehyde oxime (**122**) The crude reaction mix was separated by FCC over silica using hexane : EA (4:1) to give mixture of E and Z isomer in 70% yield.

A solution of phenyl acetaldehyde oxime (**122** , 200.0 mg, 1.50 mmol) in 4 mL DMF was cooled at 0 ºC in an ice bath and NCS (198.2 mg; 1.50 mmol) was added in portions over a period of 45 minutes. Upon completion, the ice bath was removed and continued stirring for 2 hours. 1-Thio-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (270.0 mg; 0.74 mmol) dissolved in DMF (4 mL) was added to the reaction mixture followed by triethylamine (200 µL) and stirred for another 18 h at room temperature. After 18 h the reaction mixture was diluted with diethyl ether followed by 10 mL of 0.5 M H_2SO_4 and extracted with ethyl acetate (3 × 40 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a crude reaction mixture. The crude reaction mixture was separated by FCC over silica using hexane: ethyl acetate (1:1) as the eluent to give pure 2,3,4,6-tetra-O-acetyl-β-glucopyranosylbenzylthiohydroximate (**120**) in 78% yield.

To a cooled and stirred solution of 2,3,4,6-tetra-O-acetyl-β-glucopyranosylbenzylthio hydroximate (**120**, 460.2 mg, 0.95 mmol) in anhydrous methanol (8.5 mL) under inert atm. a few drops of potassium methoxide (ca. 1M) was added until pH 11. After stirring at room temperature for 2 h the solution was neutralized by glacial acetic acid. The solvents were then evaporated under reduced pressure, the residue was dissolved in water, filtered and freeze dried to give phenyl glucosinolate (**202**) in quantitative yield.

Mp = 95-96 °C

HPLC t_R = 16.5 min (Method C).

$[\alpha]_D$ = -51.7 (c 0.181, H_2O).

UV (HPLC, MeOH-H_2O) λ_{max} (nm): 210.

^{1}H NMR (500 MHz, D_2O) δ: 7.43-7.40 (2H, m), 7.36-7.33 (3H, m), 4.70-4.68 (1 H, m), 4.03 (2H, s), 3.68-3.60 (2H, m), 3.38-3.36 (1H, m), 3.33-3.30 (2H, m), 3.26-3.23 (1H, m).

^{13}C NMR (125.8 MHz, D_2O) δ: 154.6, 136.0, 129.1, 128.0, 127.3, 81.0, 79.7, 77.0, 72.0, 68.9, 60.3, 37.9.

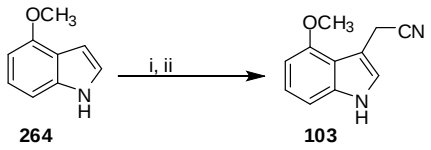
HR-ESI-MS mz calc. for $C_{14}H_{18}NO_6S$: 328.0849, found 328.0848 $[M-1]^-$.

FTIR (KBr, cm^{-1}) ν_{max}: 3341, 2883, 1607, 1562, 1494, 1412, 1270, 1050, 704.

3.2.12. 4-Methoxyindole-3-acetonitrile (103)

Magnesium turnings (20.1 mg, 0.82 mmol) in dry Et_2O (under argon) were allowed to react with iodomethane (70 μL, 1.10 mmol) at r.t. until all magnesium was

consumed (ca. 30 min). Excess Et_2O and iodomethane was distilled off and the residue was dissolved in Et_2O, a solution of 4-methoxyindole (25.0 mg, 0.17 mmol) in Et_2O (500 μL) was added drop wise to the reaction mixture under stirring at r.t. After stirring for 15 min, bromoacetonitrile (90 μL, 1.30 mmol) was added drop wise at r.t., and the mixture stirred for further 60 min at r.t. The solvent was evaporated under reduced pressure, the residue was suspended in distilled water (40 mL), and was neutralized with 2 M HCl (0.5 mL). The aqueous phase was extracted with CH_2Cl_2, the organic layer dried over $MgSO_4$, filtered, and concentrated to dryness to yield the crude product (54 mg). After FCC (CH_2Cl_2–hexane, 40:60) 4-methoxyindole-3-acetonitrile (**103**) was obtained in 52% yield (16.5 mg) (Pedras et al., 2003; Pedras et al., 2007).



264 **103**

Scheme 3.7 Synthesis of 4-methoxyindole-3-acetonitrile (**103**). Reagents and conditions: (i) CH_3I/Mg, Et_2O, r.t.; (ii) $BrCH_2CN$, r.t., 52%.

Mp = 55-56 °C

HPLC t_R= 8.0 min (method A)

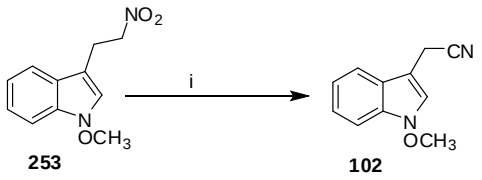
^{1}H NMR ($CDCl_3$) δ: 8.19 (1H, bs, D_2O exchangeable), 7.14 (1H, dd, J = 8.0, 8.0 Hz), 7.08 (1H, s), 6.98 (1H, d, J = 8.0 Hz), 6.53 (1H, d, J =8.0 Hz), 4.06 (2H, s), 3.93 (s, 3H)

^{13}C NMR ($CDCl_3$) δ: 154.5, 138.0, 123.9, 121.5, 119.4, 116.5, 105.4, 104.9, 100.1, 55.4, 16.2

HRMS-EI: *m/z* 186.0798 (186.0793 calc. for $C_{11}H_{10}N_2O$), EI-MS: *m/z* (relative intensity) 186.1 [M^+] (94%), 171.1 (37%), 155.1 (100%).

3.2.13. 1-Methoxyindole-3-acetonitrile (102)

To a stirred solution of 3´-(1-nitroethyl)indole (**253**, 47.0 mg, 0.21 mmol) in CH_3CN (1.5 mL) , Et_3N (300 µL, 2.13 mmol) was added followed by CS_2 (85 µL; 1.38 mmol). After stirred at 70 °C for 6 h Et_3N (100 µL, 0.71 mmol) and CS_2 (42 µL, 0.69 mmol) were added and stirred for another 6 h. After completion of the reaction, the solvents were evaporated under reduced pressure and was purified by flash column chromatography over silica using CH_2Cl_2 as the eluent to get pure 1-methoxyindole-3-acetonitirile (**102**) as brown oil (20.1 mg, 51%).



253 **102**

Scheme 3.8 Synthesis of 1-methoxyindole-3-acetonitrile (**102**). Reagents and conditions: (i) CS_2. Et_3N, 70° C, 51%.

HPLC t_R = 9.8 min (method A).

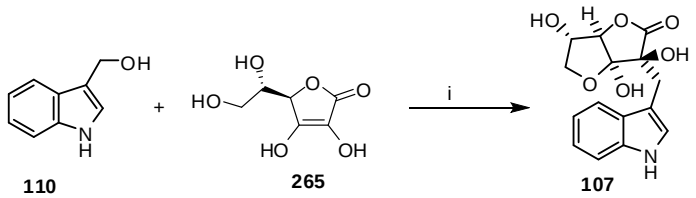
^{1}H NMR ($CDCl_3$) δ: 7.58 (1H, d, J = 8.0 Hz), 7.48 (1H, *d*, J = 8.0 Hz), 7.35-7.32 (2H, m), 7.20 (1H, dd, J = 8.0, 7.0 Hz), 4.10 (3H, s), 3.81 (2H, s)

^{13}C NMR ($CDCl_3$) δ: 132.5, 123.4, 122.5, 121.9, 120.6, 118.5, 118.1, 108.8, 100.5, 66.3, 14.4

HRMS-EI: *m/z* 186.0793 (186.0793 calc. for $C_{11}H_{10}N_2O$), EI-MS: *m/z* (relative intensity) 186.1 [M^+] (84%), 171.1 (100%).

3.2.14. Ascorbigen (107)

L-Ascorbic acid (**265**, 120.0 mg, 0.68 mmol) was added to a solution of indole-3-methanol (100.3 mg, 0.68 mmol) in phosphate buffer (pH = 4.5, 3.0 mL) and stirred for 12 h at r.t. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (2 × 30 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The crude reaction mixture was purified by flash column chromatography using DCM : MeOH (95:5) as the eluent to get pure ascorbigen (**107**, 140.3 mg, 67%) (Piironen and Virtanen, 1962).



Scheme 3.9 Synthesis of ascorbigen (**107**). Reagents and conditions: (i) NaH_2PO_4 buffer (pH = 4.5), r.t., 67% (Piironen and Virtanen, 1962).

Mp = 155-156 °C

HPLC t_R = 3.5 min (method A)

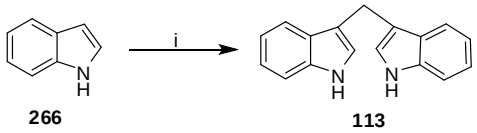
^{1}H NMR (500 MHz, D_2O) δ: 7.55 (1H, d, J = 8.0 Hz), 7.39 (1H, d, J = 8.0 Hz), 7.22 (1H, s, br), 7.15 (1H, dd, J = 7.5, 7.5 Hz), 7.05 (1H, dd, J = 7.5, 7.5 Hz), 4.29 (1H, dd, J = 4.0, 3.5 Hz), 4.09 (1H, s), 4.05 (2H, d, J = 4.0 Hz), 3.34 (1H, d, J = 14.5 Hz), 3.24 (1H, d, J = 14.5 Hz)

^{13}C NMR (125.8 MHz, D_2O) δ: 177.9, 135.8, 127.3, 126.2, 121.8, 119.3, 118.6, 111.8, 107.4, 105.7, 86.7, 79.4, 74.9, 73.3, 30.2

HRESIMS *m/z*: calc. for $C_{15}H_{14}NO_6$: 304.0826, found 304.0827.

3.2.15. Diindolyl-3-methane (113)

Indole (**266**, 200.2 mg, 1.71 mmol) was added to a solution of acetic acid (80 µL, 1.36 mmol in 2 mL water). After being stirred for 10 min, 37% aqueous formaldehyde (30 µL) was added to the reaction mixture and heated at 85-90 °C for 5 h. The mixture was cooled at room temperature, filtered, and concentrated. The crude reaction mixture was subjected to column chromatography using CH_2Cl_2 as the eluent to afford diindolyl-3-methane (**113**, 200.1 mg, 95% yield) (Jackson et al., 1987).



266 113

Scheme 3.10 Synthesis of diindolyl-3-methane (**113**). Reagents and conditions: (i) HCHO, AcOH, r.t., 95%.

Mp = 167-168 °C

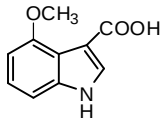
HPLC: t_R = 16.1 min (method A)

^{1}H-NMR (500 MHz, $CDCl_3$) δ: H 7.88 (1H, s, br), 7.65 (1H, d, *J* = 8.0 Hz,), 7.37 (1H, d, *J* = 8.0 Hz), 7.21 (1H, dd, *J* = 8.0, 7.0 Hz), 7.12 (1H, dd, *J* = 8.0, 7.0 Hz), 6.94 (1H, d, *J* = 1.0 Hz), 4.27 (1H, s,)

^{13}C NMR (125.8 MHz, $CDCl_3$) δ: 136.7, 127.8, 122.4, 122.1, 119.4, 119.3, 115.9, 111.2, 21.4

HREIMS *m/z*: calc. for $C_{17}H_{14}N_2$: 246.1157, found 246.1162 (% relative abundance) measured: 246.1 (100%), 117.1 (77%).

3.2.16. 4-Methoxyindole-3-carboxylic acid (239)



239

Indole-3-carboxaldehyde (**47**, 100.2 mg, 0.69 mmol) was added to a solution of thallium trifluoroacetate (562.1 mg, 1.03 mmol) in TFA (1.2 mL), and the mixture was stirred at 30 °C for 3h. After evaporation of the solvent under reduced pressure, I_2 (525.8 mg, 2.07 mmol), CuI (525.8 mg, 2.76 mmol), and DMF (5.0 mL) were added to the residue. After stirred at r.t. for 1h, $NaOCH_3$ (15% w/v, 5 mL) was added and the stirring was continued at 100-110° C for 3 h. The reaction was cooled to room temperature, diluted with CH_2Cl_2-MeOH (95:5) and filtered through celite. The filtrate was washed with water and brine, dried, and concentrated under reduced pressure. Purification of the product by FCC over silica using EtOAc: hexane (3:1) as the eluent yielded pure 4-methoxyindole-3-carboxaldehyde (**248**, 62.1 mg, 75%).

A solution of $NaHClO_2$ (870.5 mg, 8.0 mmol) and $NaH_2PO_4.2H_2O$ (940.2 mg, 6.0 mmol) in H_2O (4.2 mL) was added to a solution of 4-methoxyindole-3-carboxaldehyde (**248**, 70.2 mg, 0.40 mmol) in t-BuOH (4.2 mL) and 2-methyl-2-butene (4.2 mL) at 0 °C. After stirring at r.t. for 48 h, the reaction mixture was extracted with Et_2O (2 × 30 mL), dried and the product was purified by FCC over silica using CH_2Cl_2-CH_3OH (99:1), to yield 69.5 mg (91%) of the desired compound **239** (Somei et al., 2001).

Mp = 181-182 °C

HPLC t_R = 5.2 min (method A)

^{1}H NMR (500 MHz, CD_3CN) δ: 11.6 (1H, s), 10.2 (1H, s, br), 7.99 (1H, d, J = 3.0 Hz), 7.24-7.20 (2H, m), 6.84-6.80 (1H, m), 4.09 (3H, s)

^{13}C NMR (125.8 MHz, CD3CN) δ: 165.0, 152.0, 139.7, 135.1, 125.3, 114.9, 109.1, 108.4, 103.8, 57.7

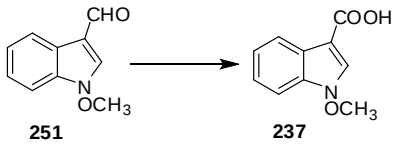
HREIMS *m/z*: calc. for $C_{10}H_9NO_3$: 191.0582, found 191.0583; *m/z* (%): 191.1 $[M]^+$ (100), 172.0 (12), 162.1 (8), 144.0 (33), 132.0 (17), 118.1 (15), 104.1 (64).

UV (HPLC, MeOH-H_2O) λ_{max} (nm): 210, 230, 290

FTIR (KBr, cm^{-1}) ν_{max}: 3183, 1696, 1526, 1395, 1245, 1089, 740, 735.

3.2.17. 1-Methoxyindole-3-carboxylic acid (237)

A solution of $NaHClO_2$ (620.0 mg, 5.72 mmol) and $NaH_2PO_4.2H_2O$ (670.1 mg, 4.29 mmol) in H_2O (3.0 mL) was added to a solution of 1-methoxyindole-3-carboxaldehyde (**251**, 50.2 mg, 0.29 mmol) in t-BuOH (3.0 mL) and 2-methyl-2-butene (3.0 mL) at 0 °C. After stirring at r.t. for 48 h, the reaction mixture was extracted with Et_2O (2 × 30 mL), dried and the product purified by FCC over silica using CH_2Cl_2-CH_3OH (99:1), to yield 50.4 mg (91%) of the desired compound **237** (Somei et al., 2001).



251 **237**

Scheme 3.11 Synthesis of 1-methoxyindole-3-carboxylic acid (**237**). Reagents and conditions: (i) $NaHClO_2$, NaH_2PO_4. $2H_2O$, 2-methyl-2-butene, 92%.

Mp = 165-166 °C

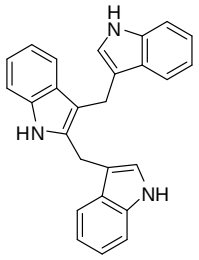
HPLC t_R = 6.2 min (method A)

^{1}H NMR (500 MHz, CD_3CN) δ: 8.14 (1H, s), 8.11 (1H, d, J = 8.0 Hz), 7.55 (1H, d, J = 8.5 Hz), 7.34 (1H, dd, J = 7.0, 7.0 Hz), 7.28 (1H, dd, J = 8.0, 8.0 Hz), 4.15 (3H, s)

^{13}C NMR (125.8 MHz, CD_3CN) δ: 167.0, 133.5, 130.9, 124.8, 124.3, 123.8, 122.6, 110.3, 103.7, 68.1

HREIMS *m/z*: calc. for $C_{10}H_9NO_3$: 191.0582, found 191.0579; *m/z* (%): 191.1 $[M]^+$ (100), 176.0 (20), 132.0 (75).

3.2.18. [2-(Indol-3-ylmethyl)-indol-3-yl]indole-3-ylmethane (243)



243

HPLC t_R = 20.6 min (method A)

^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.04 (1H, s, br), 7.88 (1H, s, br), 7.77 (1H, s, br), 7.71 (1H, d, J = 8.0 Hz), 7.53 (1H, d, J = 7.5), 7.42-7.36 (3H, m), 7.22-7.18 (3H, m), 7.11-7.02 (4H, m), 7.01 (1H, s), 6.80 (1H, s), 4.34 (2H, s), 4.31 (2H, s)

HREIMS *m/z*: calc. for $C_{26}H_{21}N_3$: 375.1735, found 375.1734; *m/z* (%): 375.2 $[M]^+$ (30), 258.1 (100), 217.1 (8), 130.1 (34).

3.3. Mycelial radial growth and antifungal bioassays

Three-day-old cultures of *S. sclerotiorum,* 4-day-old cultures of *R. solani,* and 7-day-old cultures of *A. brassicicola* grown on PDA under constant light at 23 ± 1º C were used for mycelial radial growth assays. Plugs (4 mm) were cut from the edges of mycelia and placed inverted into six-well agar plates (for *S. sclerotiorum* and *R. solani*) or twelve-well agar plates (*A. brassicicola*) amended with test compounds (dissolved in CH_3CN, DMSO, or H_2O). The final concentrations of each compound in agar varied from 0.10 to 0.50 mM, with a CH_3CN or H_2O concentration of 1%. The plates were allowed to grow under constant light at 23 ± 1º C; the diameter of the mycelial mat was measured after 24 h (*S. sclerotiorum*), 48 h (*R. solani*) and 96 h (*A. brassicicola*) and compared to control mycelia grown on plates containing CH_3CN, DMSO, or H_2O only.

3.4. Metabolism of phytoalexins and phytoanticipins

3.4.1. Metabolism of phytoalexins

Liquid cultures of *B. cinerea* were grown in 250 mL Erlenmeyer flasks containing 100 mL of minimal medium inoculated with six mycelial plugs (6 mm diameter) per 100 mL, and cultures were incubated on a shaker at 120 rpm. After 72 h at 23 ± 1 °C under constant light, a solution of the phytoalexins (**33** and **39**) or compounds (**225**, **226**, **49**, and **50**) in CH_3CN (250 µL) was added to the cultures, for a final concentration of 0.10 mM. The flasks were returned to the shaker, and samples (5 mL) were withdrawn at various times and either extracted immediately with EtOAc (10

× 2 mL) or immediately frozen. The EtOAc residue was dissolved in MeOH or CH_3CN and analyzed by HPLC (method A).

3.4.2. Metabolism of phytoanticipins (aryl and indolyl glucosinolates and derivatives by plant pathogenic fungi)

Liquid cultures of each pathogen were grown in 250 mL Erlenmeyer flasks containing 100 mL of minimal medium inoculated fungal spores for a final concentration of 1×10^6/100 mL for *A. brassicicola*, or six mycelial plugs (6 mm diameter) per 100 mL for *R. solani* and *S. sclerotiorum*, and cultures were incubated on a shaker at 120 rpm. After 48–72 h at 23 ± 1 ºC, under constant light, the cultures were filtered aseptically and the mycelia washed with sterile water. Then the mycelia from each culture were transferred to 250 mL flasks containing sterile water (100 mL), and a solution of each compound in sterile water or CH_3CN (100-250 µL depending on solubility) was added to the cultures, for a final concentration of 0.10 mM. Similar control solutions containing compounds only or mycelia were prepared. The flasks were returned to the shaker, and samples (5-10 mL) were withdrawn at various times, immediately frozen, and lyophilized. The residue was dissolved in MeOH, or MeOH-H_2O and analyzed by HPLC, using method A for compounds (**40**, **102**, **103**, **110**, **113**, **170**, and **185**), and method B for compounds (**65**, **66**, **86**, **87**, and **90**) and method C for compounds (**107**, **159**, **202**, **228**, and **233**), as described previously (Pedras et al., 2010).

3.5. Myrosinase activity

Myrosinase activity of in crude cell free extracts of *A. brassicicola, R. solani* and *S. sclerotiorum* was determined as follows.

3.5.1. Preparation of crude cell free extracts

Four to five-day-old mycelia of *A. brassicicola* (1×10^6 spores/100 mL MM), *R. solani* and *S. sclerotiorum* (6 plugs of 6 mm/100 mL) were filtered, washed and dried under vacuum, weighed and frozen immediately. The frozen mycelia (4-5 g) were ground with NaH_2PO_4 buffer (pH 7.0, 8-10 mL) at 4 °C, centrifuged twice (2400 × g for 30 min and 11500 × g for 25 min), the supernatant (cell-free extract) was dialyzed in NaH_2PO_4 buffer (pH 7.0, 100 mL) for 3 hrs and used for myrosinase activity assays.

3.5.2. Preparation of BSA calibration curve

The Bradford protein assay was used to estimate the quantities of proteins in the cell homogenate using a calibration curve prepared from bovine serum albumin (BSA). A stock solution (1 mg/mL) of BSA in the extraction buffer was prepared from which five other concentrations (0.30, 0.25, 0.20, 0.15, and 0.10 mg/mL) were prepared by serial dilution using the same buffer. In a spectrophotometric cell (1 mL) were taken 100 µL of each solution and 1 mL of Bradford reagent. After mixing, the solution mixture was incubated for 5 min and the optical density was measured at 595 nm. A blank sample containing 100 µL extraction buffer and 1 mL Bradford reagent was used

as control. All samples were prepared in triplicate and finally the calibration curve was obtained by plotting concentration vs. optical density.

3.5.3. Protein measurements

40 µL of cell homogenate was diluted to 1 mL using the extraction buffer. In a spectrophotometric cell (1 mL) were taken 100 µL of this diluted solution and 1 mL of Bradford reagent. After mixing, the solution mixture was incubated for 5 min and the optical density was measured at 595 nm. A blank sample containing 100 µL extraction buffer and 1 mL Bradford reagent was used as control. All samples were prepared in triplicate and finally the concentration of proteins was determined using the BSA calibration curve.

3.5.4. Enzyme assay

Glucosinolates (250 µL, 10 mM) in buffer (KH_2PO_4, pH 7.0) was added to the cell-free extract (720 µL) and 30 µL of ascorbic acid (10 mM). After 90 minutes incubation, 200 µL was used to analyze glucose content with an enzymatic (hexokinase-based) glucose test kit (Sigma). The specific activity was calculated as the number of nmoles of product (glucose) formed per mg of protein per min. The amounts of product formed were quantified using calibration curves built with authentic synthetic samples. The Bradford assay method was used to calculate the protein content of cell-free extracts using a standard BSA calibration curve (wavelength 595 nm). Commercially available myrosinase from *S. alba* was dissolved in buffer (pH 7.0) in 0.05 mg/mL Glucosinolates in buffer (250 µL, 10 mM) was added to the commercial

myrosinase solution (720 µL) and 30 µL of ascorbic acid (10 mM). After 90 minutes incubation, 200 µL was used to analyze for glucose content with an enzymatic (hexokinase-based) glucose test kit (Sigma). Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalyzed by hexokinase. Glucose-6-phosphate (G6P) is then oxidized to 6-phospho-gluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalyzed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to glucose concentration.

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APPENDIX

One-way ANOVA

The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means of three or more independent (unrelated) groups. Once one-way ANOVA or a repeated measure of ANOVA has been performed and a statistically significant difference is observed. ANOVA is a hypothesis in which ratio of variance between data sets to variance within a data set is used to generate a test statistic. The variance of set of n values (X1, X2,…….Xn) is given by the following equation.

$$\frac{\sum_{i=1}^{n}(Xi - X)^2}{\text{n -1}}$$

Where, the sum of squares (SS) = $\sum_{i=1}^{n}(Xi - X)^2$ and the degrees of freedom = n-1

If the variance between different data sets is large compared to the variance within a data set, then it is likely that data sets are different. The test statistic F is equal to the between treatment square divided by the error mean square or can be expressed as below where

$$F = \frac{\text{Between group variance}}{\text{Within group variance}}$$

If there is no difference in the factors, then F = 1.

An example of ANOVA analysis of bioassay results of six compounds angaint *A. brassicicola.*

A. brassicicola						
	IAN	1-IAN	4-IAN	Ascorbigen	I-3-Methanol	DIM
1	25	45	62	38	46	100
2	25	42	58	36	41	100
3	32	42	58	34	41	100
n	3	3	3	3	3	3
X	27.333	43.000	59.333	36.000	42.667	100.000
s	4.041	1.732	2.309	2.000	2.887	0.000
X_{ave}	51.389					

source	df	SS	MS	F	P-value
treatments	5	10164.278	2032.856	329.6523	-0.0003
error	12	74.000	6.167		
total	17	10238.278			

The P- value is less than 0.05, which tells that these group data, is significantly different. However, the ANOVA analysis simply indicates there is a difference between two or more group means, but it does not tell which means are significantly different. Tukey's multiple comparison test is one of several tests that can be used to determine which means amongst a set of means differ from the rest. Tukey's multiple comparison test is also called Tukey's honestly significant difference test or Tukey's HSD. The test compares the difference between each pair of means with appropriate

adjustment for the multiple testing. The results are presented as a matrix showing the result for each pair, either as a P-value or as a confidence interval.

Tukey HSD Test

The Tukey HSD (honestly significant difference) test is a pair wise comparison of every combination of group pairs. This test calculates the mean difference for each treatment or group pair, calculates a q test statistic for each pair, and displays the P value for that comparison. Tukey HSD test controls the errors of all comparisons simultaneously.

The following values are calculated when performing a Tukey HSD post hoc test.

Tukey's HSD was designed for a situation with equal sample sizes per group, but can be adapted to unequal sample sizes as well.

The formula for Tukey's is:

$$HSD = F \sqrt{MSE / n}$$

Where,

MSE = Mean Square Error from ANOVA table

n = number of replicates per treatment

Tukey's HSD value calculated from the above formula shows how far apart any two means must be in order them to be significantly different. If the difference between means is equal to or greater than HSD, then it's significant. If the difference between means is less than the HSD value, then it's not a significant difference.

Mean Difference - This value represents the difference between the means of the groups or treatments being compared.

F - This value is the F test statistic. Large values of F indicate that the difference of the two treatments or groups being compared is statistically significant.

P value - This value represents the P value that corresponds to the F test statistic. It determines if there is a statistically significant difference between the treatments or groups being compared. If this value is below a certain level (usually 0.05), the conclusion is that there is a difference between the groups.

References:

One Way ANOVA, Two Way ANOVA, Tukey HSD, Student-Newman-Keuls, and Scheffe Tests

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